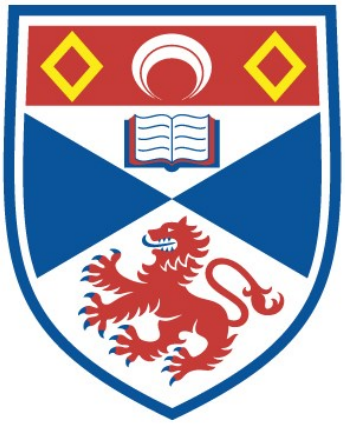
PURIFICATION AND IMMOBILISATION OF URICASE FOR USE IN AUTOMATED ANALYSIS

Abu Bakar bin Salleh

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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FOR USE IN AUTOMATED ANALYSIS

by

Abu Bakar bin Salleh

A thesis submitted to the University of St. Andrews in application for the degree of Doctor of Philosophy.

University of St. Andrews,
Department of Biochemistry and Microbiology,
North Street,
St. Andrews,
Scotland.

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A procedure for the purification of uricase from porcine liver is described, utilising the technique of bioaffinity chromatography as a major purification step. Bioaffinity support is prepared by coupling of urate to bisoxirane-activated Sepharose 4B. Purified uricase shows a single protein band corresponding to the activity band, when applied to polyacrylamide disc-gel electrophoresis. A single protein band but no activity band is obtained by SDS-acrylamide disc-gel electrophoresis. The enzyme has a pH optimum in the range of 8.9-9.1, with a V_{max} of 12.6 $U.mg^{-1}$, a K_m of 1×10^{-5}M and a molecular weight of 13×10^4. Each enzyme molecule comprises 4 subunits of molecular weight $32\text{-}34 \times 10^3$ each. Nylon tube is directly activated by alkaline glutaraldehyde solution to generate reactive centres for enzyme immobilisation. The optimal conditions for activation are studied. Purified uricase is immobilised to PEI-glutaraldehyde-nylon tube with about 20% activity retention. The derivatised enzyme has a pH optimum in the range of 9.0-9.2 and a K_m of about 4 times that of the soluble enzyme. Immobilised uricase is incorporated into a continuous flow Auto-Analyser for use in the automated analysis of serum urate. For this purpose, the immobilised enzyme shows good storage and operational stability. Linear calibration plots can be obtained for a urate range of 2 - 20 $mg.100ml^{-1}$, the method exhibiting a high degree of precision and accuracy. The results obtained also compare favourably with an established method of urate assay which employs soluble uricase.

Declaration

I hereby declare that the following thesis is based on work performed by me, that the thesis is my own composition and that no part of it has been presented for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. W.E. Hornby and Dr. W.M. Ledingham.

Certificate

I hereby declare that Abu Baker bin Salleh has spent at least nine terms engaged in research work under the supervision of Dr. W.E. Hornby and myself, and that he has fulfilled the conditions of Ordinance General No: 12 and the Resolution of the University Court 1967, No: 1, and that he is qualified to submit the accompanying Thesis for the degree of Doctor of Philosophy.

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Abbreviations

BIS	-	N,N-methylenebisacrylamide
EDTA	-	ethylene-diamine-tetra-acetate
PEI	-	polyethyleneimine
SDS	-	sodium dodecyl sulphate
TEMED	-	N,N,N',N'-tetramethylenediamine
TRIS	-	tris(hydroxymethyl) aminoethane

C O N T E N T S

1. INTRODUCTION

1.1 Uric Acid in serum and methods for determination of its levels

Uricase, also known as urate oxidase (urate:oxygen oxidoreductase, EC 1·7·3·3) is responsible for the oxidative scission of the purine ring. It is of great importance to all organisms that do not excrete uric acid or some other purine. It is generally accepted that all mammals except man and the higher apes possess uricase (1).

Due to the absence of uricase, man excretes uric acid as the final product of purine catabolism. Thus a level of urate is naturally maintained in the blood. Orowan (2) suggested that the presence of urate in the blood has contributed in the evolution of man as an intellectual animal. Urate, like other purines such as caffeine and theobromine, may have acted as a cerebral stimulant, and the constant pressure of stimulation may have promoted cerebral development. It is believed that there may have been a gradual elimination of uricase during evolution. Studies in some species of Old World monkeys showed the presence of highly unstable uricase, unlike that of prosimians and rabbits, which perhaps reflected a partial degeneration of the enzyme in these species (3).

On the other hand the loss of uricase may be considered as an unfortunate occurrence during evolution of man and it is held responsible for the occurrence of hyperuricaemia and related diseases.

Hyperuricaemia is rather difficult to define as it depends on what is considered as the normal level of urate in serum. Studies of different population samples have shown that the mean values of the levels of urate in serum vary with age, sex, environmental, ethnic and anthromorphic differences (4). A typical study of a healthy population showed the urate levels in serum to be 5.1 ± 0.8 mg uric acid.100 ml^{-1} in men and 4.1 ± 0.7 mg uric acid.100 ml^{-1} in women (5). Values of serum urate greater than 7 mg.100 ml^{-1} in men and 6 mg uric acid.100 ml^{-1} in women are chosen as the arbitrary values to define hyperuricaemia, based on epidemiological surveys (6). Peters and Van Slyke (7) considered 6.4 mg uric acid.100 ml^{-1} as the upper limit of 'normal' level of urate in serum. At this concentration, the serum is saturated with monosodium urate and urate crystals may precipitate.

The relative insolubility of uric acid and the absence of uricase to degrade uric acid to the more soluble compound, allantoin, gives rise to a disease known as gout. The accumulation of urate above the normal level in serum may lead to the typical gout syndrome, such as recurrent attacks of acute arthritis, formation of tophaceous deposits of monosodium urate within joint tissues and production of stones in the kidneys.

Primary gout is a genetically determined disorder of purine metabolism found in men. There are some rare occurrences in women usually found in post-menopausal individuals. It seems that sex differences in the levels of urate in serum disappear with age, and apparently metabolic and hormonal factors are involved.

Primary gout may be due to the increased production of uric acid brought about by increased purine biosynthesis *de novo*, increased catabolism of nucleic acids or ingestion of excessive purine diet. Decreased excretion of uric acid caused by decreased renal clearance also contributes to the increase in serum urate levels. These factors may act singly but more often all at once in contributing to the hyperuricaemic state and gout.

Secondary gout is an acquired form of the disease. Gouty arthritis is developed as a complication of hyperuricaemia caused by disorders such as leukemia, uronic nephritis and polycythaemia. These types of diseases are usually due to high urate levels caused by abnormally rapid turnover of nucleic acids.

Gout has been known for centuries and the disease described in ancient literature (8). More up to date and detailed treatises are available (4,6,9).

A unique form of secondary gout may be found in children and adolescents but its occurrence is very rare. However, another disorder attributed to excessive production of uric acid, affecting children only, is the Lesch-Nyhan syndrome (10,11). This disease is an X-linked disorder and thus affects men only. The syndrome is characterised by muscle spasticity, mental retardation and compulsion for self-mutilation. The first sign of abnormality, delay in motor development, can be observed as

early as four months after birth, and this disorder usually leads to death before puberty.

The Lesch-Nyhan syndrome is attributed to a deficiency in a particular enzyme in the purine biosynthetic pathway, hypoxanthine-guanine phosphoribosyl transferase (HPRT) (IMP: pyrophosphate phosphoribosyl transferase, EC 2.4.2.8) (12). Fig. 1.1.1 shows the biosynthetic pathway of purines and its feedback control. Purine nucleotides and their pyrophosphates normally serve to inhibit 5-phosphoribosyl-1-pyrophosphate (PRPP)-glutamine amidotransferase (ribosylamine-5-phosphate : pyrophosphate phosphoribosyl transferase (glutamate amidating), EC 2.4.2.14), the rate limiting enzyme in the pathway. HPRT, the enzyme deficient in the Lesch-Nyhan syndrome shares the same substrate as the PRPP-glutamine amidotransferase. The absence of HPRT, results in the accumulation of PRPP. Increased PRPP concentration is found in children suffering from this disease and it appears to contribute to excessive purine synthesis.

Restriction of purines, proteins, alcohols and caloric intake has been used to regulate urate levels in serum. Influences upon both the rate of production and excretion of urate have been cited, but the full role of their complex inter-relationship is not known. Thus dietary therapy is no longer recognised as an acceptable treatment.

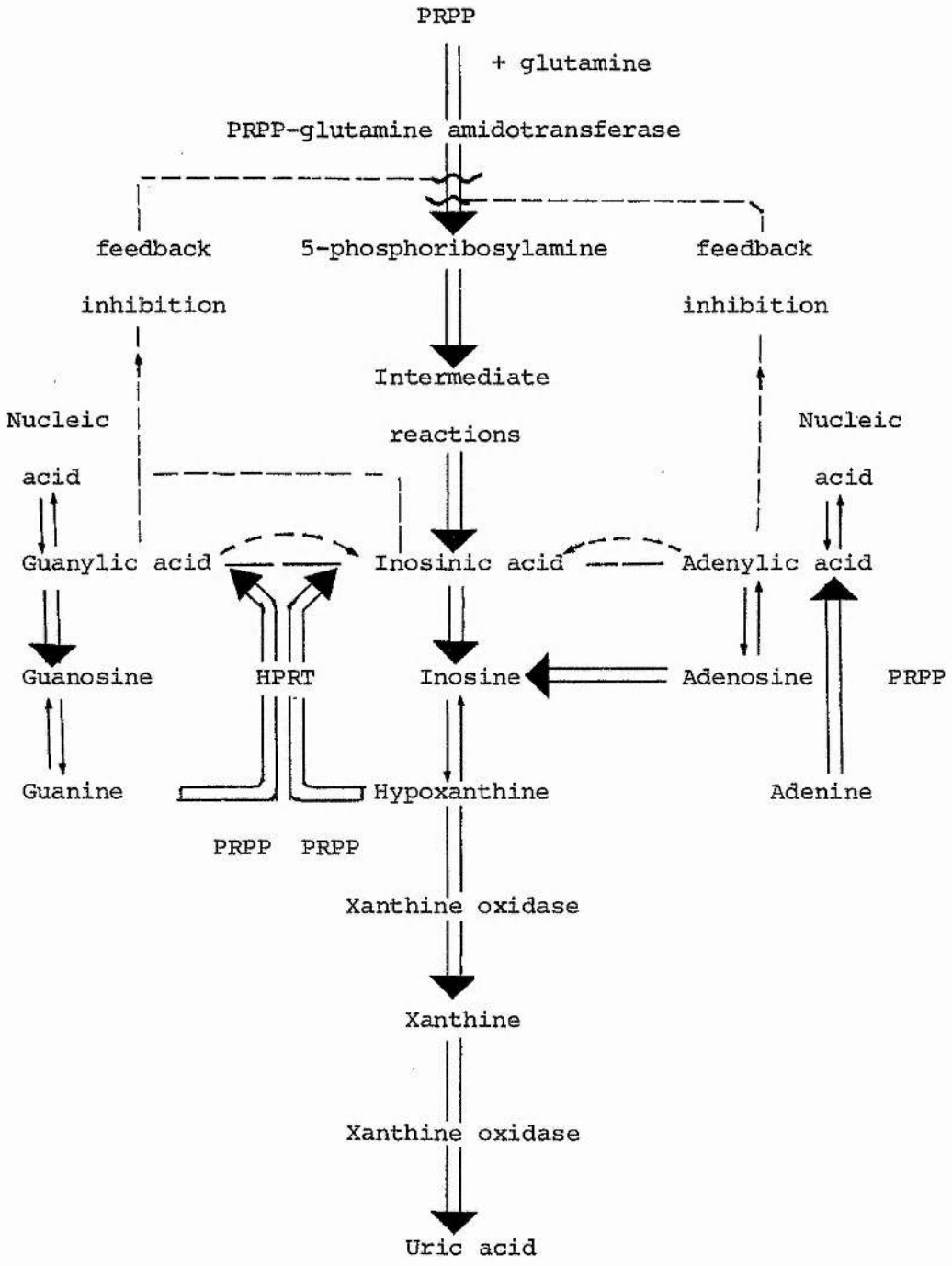


Fig. 1.1.1 Feedback control of purine biosynthesis.

Colchicine is commonly used for treatment of acute gout. The mechanism of action is uncertain but it does relieve the pain of arthritic attacks. Other drugs such as phenylbutazone, oxyphenbutazone and indomethacine are available for use in the therapy of acute gout (13).

Hypouricaemic drugs provide the definitive method for controlling the disorders associated with gout (14). Uricosuric drugs such as probenecid and sulphinpyrazone have been widely used, while combined action drugs such as halofenate, a hypo-lipidaemic and uricosuric drug, and ticrynafen, a diuretic urico-suric drug, have found limited acceptance.

Allopurinol has been used extensively to decrease uric acid synthesis. Fig. 1.1.2 shows its possible mechanism of action. It is worth noting that since step 1, 2 and 3 the rate inhibition steps are dependent on HRPT, allopurinol has no effect in decreasing *de novo* synthesis of purines in patients suffering the Lesch-Nyhan syndrome, resulting in striking increase in excretion of hypoxanthine and xanthine which roughly equals the decrease in uric acid formation.

Infusion of soluble uricase has been shown to bring about a decrease in serum urate levels (15-17). However, this method is ineffective mainly due to the formation of antibody against the enzyme. The use of immobilised uricase for this purpose is very much in the experimental stage (18,19).

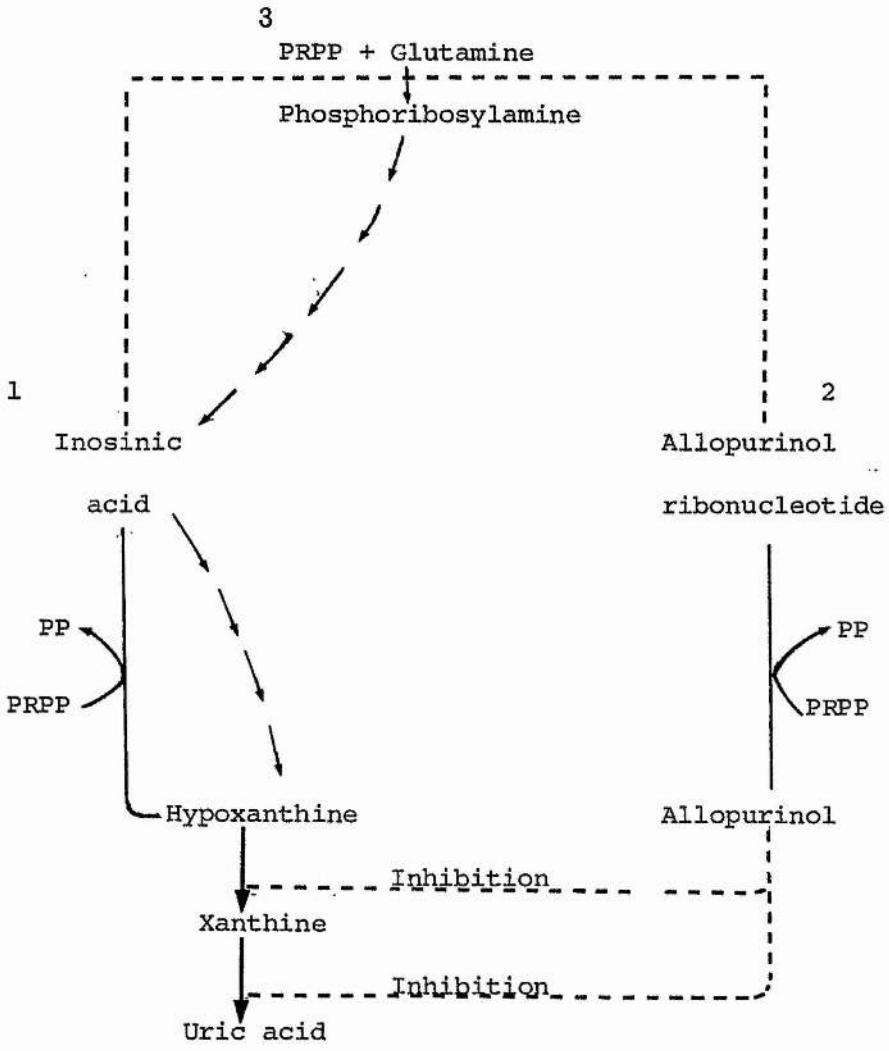


Fig. 1.1.2 Possible mechanism of allopurinol action in inhibition of purine synthesis and lowering of urate levels in serum.

Unfortunately in the case of the Lesch-Nyhan syndrome, the lowering of urate levels in serum is not sufficient in halting damage to the nervous system and mental retardation even though the disorder is discovered in the initial stages. No cure has been discovered to combat this disorder effectively (4).

Many methods for determining urate levels in biological fluids have been developed for use in diagnostic purposes and monitoring therapy (20). Traditional methods are based on either the reduction of phosphotungstate (21) or copper-complex (22) which are subject to interferance due to turbidity and non-urate reducing substances such as glucose, cysteine and ascorbic acid (23). Thus methods utilising uricase are generally recognised to be superior (24,25). Enzymatic methods utilising differential ultra-violet spectrophotometry have been developed, urate being maximally absorbed at 290-293nm (26-28). Alternatively hydrogen peroxide produced by the uricase catalysed reaction can be measured, as shown by the method of Kageyama (29).

$$\text{Uric Acid} + 2H_2O + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + CH_3OH \xrightarrow{\text{catalase}} HCHO + 2H_2O$$

$$HCHO + \text{acetylacetone} + (NH_4^+) \longrightarrow \text{3,5-diacetyl-1,4-dihydrolutidine} + 3H_2O$$

Hydrogen peroxide reduced in the presence of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) is coupled to a condensation reaction which yields a colour product determined at 410nm.

Uricase-generated hydrogen peroxide can also be oxidatively couples to selected chromogens, catalysed by peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) to produce a colour product. The choice of the chromogens is also critical as some of the chromogens are affected by reducing compounds (30).

The use of chromogens can be avoided by linking to a third enzymic reaction. Catalase-generated aldehyde is reacted with NAD or NADH in the presence of aldehyde or alcohol dehydrogenase (aldehyde: NAD(P) oxidoreductase, EC 1.2.1.5 or alcohol:NAD oxidoreductase, EC 1.1.1.1). The absorption of NADH or NAD is a measure of urate concentration (31,32).

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + 2H_2O$$

(a) $$\text{Acetaldehyde} + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{aldehyde}} \text{acetate} + NADH + H^+$$

(b) $$\text{Acetaldehyde} + NADH + H^+ \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} \text{ethanol} + NAD^+$$

Since the development of automated analysers (33), methods utilising this procedure are preferred due to their greater precision and speedier operation. The phosphotungstate method has been incorporated onto the AutoAnalyser (Technicon) (34) and Morgenstern (35) adopted the copper chelation method onto the AutoAnalyser and the Robot Chemist (Warner-Chillot Laboratories). Gochman and Smitz (30) developed a uricase-peroxidase method incorporated onto the AutoAnalyser, and the method of Kageyama has also been adapted to an automated procedure (36).

MacRae (37) automated a uricase method utilising urate absorption at 290nm.

The problems associated with the use phosphotungstate and copper chelation techniques in manual methods are still potentially present when these methods are adapted to automated procedures (23). Generally these methods give higher values of urate levels when compared with enzymatic methods (23,38,39).

1.2 Purification of uricase

1.2.1 Methods for purification of uricase and some studies on the enzymic reaction of uricase

Uricase was first identified some seventy years ago by Batelli and Stern (40). Since then there have been many attempts at isolating the enzyme and studying its properties.

Uricase has been isolated from various sources, including porcine liver (41-43), bovine kidney (44-46), rat liver (47), bacteria (48,49) and yeast (50). Baudhuin et al. (51) studied extensively the intracellular localisation of uricase in rat kidney and liver, and found that this enzyme was located in the cytoplasmic particles known as microbodies and peroxisomes. Leighton et al. (52) and Tsukada et al. (53) clarified that uricase was in fact localised in the crystalloid core of the microbodies.

In many procedures (41-43), the initial stage was to obtain the mitochondrial fraction from neutral pH suspension, a step requiring high speed centrifugation. Leone (44) and London and Hudson (45) used alkaline extraction of uricase in the initial step. In fact the concept of extraction or precipitation of uricase in alkaline or acidic condition is adopted in many preparative procedures as exemplified by the method of Robbin et al. (54).

Adsorption chromatography was used by some workers with calcium phosphate gels being the early choice (42,44,45). Later, the use of ionic exchangers was reported but the main result seemed

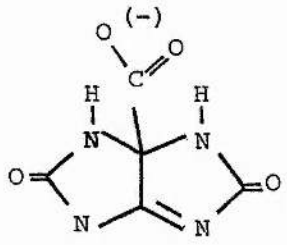
to be low recovery of enzyme activity (45,48-50).

Low enzyme recovery was also reported when tert-butanol was used as a precipitating agent (42,43,47).

Mahler (42) obtained an enzyme with specific activity of about 10 $U.mg^{-1}$, but with recovery between 10-30%. Leone (45) achieved less than 30% recovery with uricase of specific activity of around 0.6 $U.mg^{-1}$. Townsend and Lata (47) achieved nearly 50% recovery with the specific activity of uricase of nearly 10 $U.mg^{-1}$, obtained from rat liver. On the other hand, Yokota (41) only managed to recover 36% uricase activity from porcine liver, with specific activity of around 5 $U.mg^{-1}$.

Baum et al. (55,56) and Mahler (57) studied uricase purified from porcine liver, and showed evidence that the enzyme contained copper. Studies with models of copper-catalysed oxidation of urate, suggests that copper is an essential component of the catalytic site of uricase.

Uricase does not catalyse the conversion of urate directly to allantoin, carbon dioxide and water. Praetorius (58) and Mahler et al. (59) reported the appearance of transitory intermediates, and structure 1 has been proposed for one of these intermediates, 1-carboxy-2,4,5,8-tetraazabicyclo(3.3.0)octa-4-ene-3,7-dione.



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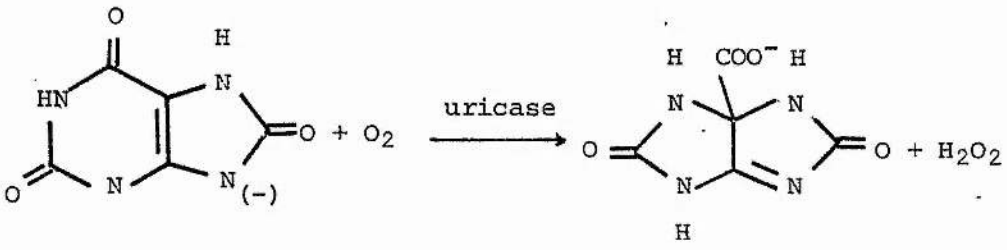
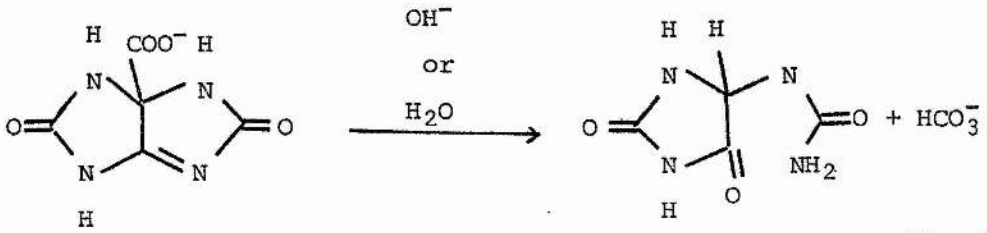
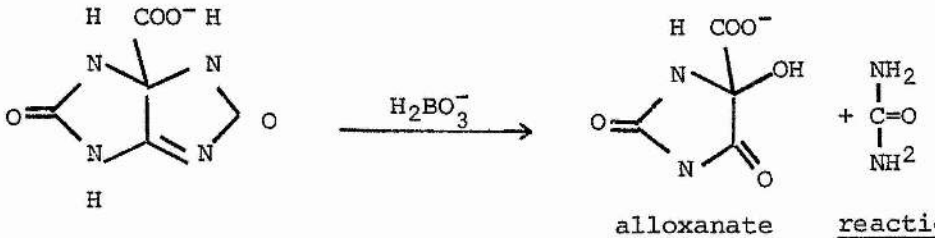
The proposed structure assumes an oxidation by O_2 during the course of the enzyme catalysed reaction. Klemperer (60) suggested the CO_2 was released during the intermediate breakdown, and this has been substantiated by Pitts and Priest (61). Thus it appears that urate is catalysed to an intermediate by uricase (reaction 1). The decay of intermediate is independent of O_2 and uricase, and the product can vary depending upon the absence (reaction 2) or presence (reaction 3) of borate (62).



reaction 1

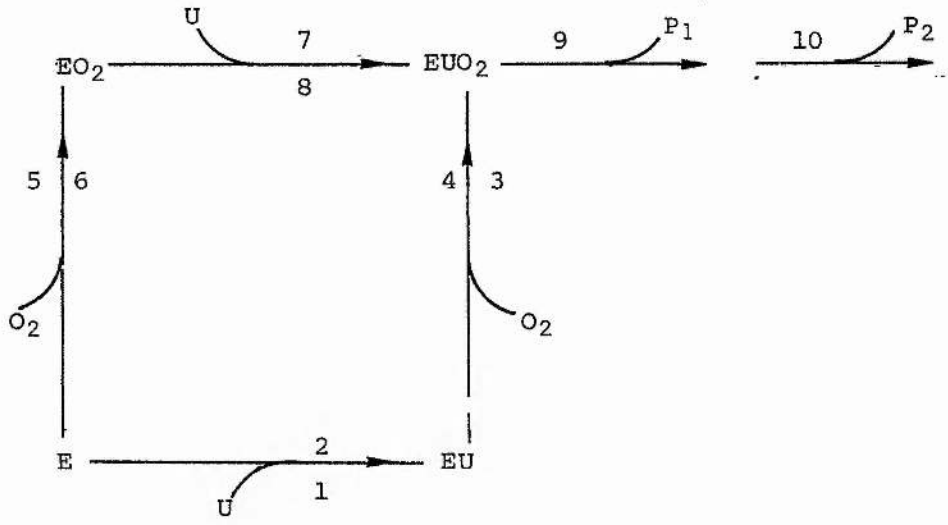


reaction 2



reaction 3

In their investigation of steady state mechanism of uricase, Baum et al. (56) proposed an ordered sequential binding of substrate, oxygen and urate, based on the observation that the apparent K_m and V_{max} are different in air and in 100% oxygen atmosphere. Recent studies however suggests random binding is more likely (63).



The scheme above was proposed by Pitts and Priest (63). It depicts a single-site model for the uricase enzymic reaction. E designates the free or unbound enzyme, U designates urate. Reaction steps 2,4,6 and 8 are the reverse of reaction 1,3,5 and 7. Steps 9 and 10 show irreversible reactions, however, there is no suggestion that these steps depicts the mechanism for product release.

1.2.2 Bioaffinity chromatography

Bioaffinity chromatography exploits a unique biological property of many proteins in their ability to bind ligands specifically and reversibly. Thus isolation of proteins by bioaffinity chromatography presents considerable advantages over conventional procedures for protein purification which are generally based on relatively small differences in physico-chemical properties of the protein mixtures.

The principle of biospecific adsorption was initially used in the isolation and purification of antibodies with solid phase immuno-adsorbents (64). Now its use is more widespread, and in principle this technique can also be applied to purify enzymes, nucleic acids, hormones or hormone receptors and drugs (65-68).

Successful application of this technique depends largely on the choice of support matrix and ligand specificity, the means of linkage between ligand and support, and the conditions used for adsorption and elution.

An ideal support matrix for bioaffinity chromatography of enzyme must have low interaction with proteins in general to minimise non-specific adsorption of proteins. It must possess good flow properties that can be retained even after ligand coupling, and naturally the integrity of the matrix must be maintained during chemical activation and coupling to a variety of ligands. Furthermore, it should possess wide stability

spectrum mechanically and chemically to the various conditions of pH, ionic strength, temperature, or the presence of denaturants which may be utilised in the chromatographic process.

Cuatrecasas (69) discussed in detail the merit of many insoluble supports, and concluded that the beaded derivatives of agarose, a polysaccharide polymer, was ideal in many ways for use as an affinity support.

The small molecule chosen for attachment to the support must display specific affinity for the macromolecule to be purified. It can be an inhibitor, a cofactor or in some cases a substrate. The small molecule must possess chemical groups that can be modified for linkage to the support.

The affinity of enzyme for the ligand can be affected by steric hindrance if the ligand is attached in close proximity to the support. To overcome possible steric hindrance, ligand is attached at a distance from the support by introducing a spacer molecule between the ligand and support (69). Long hydrocarbon chains can be used as the spacer molecule between the support and ligand, either by preparing the ligand with the spacer molecule attached before coupling to the support, or the spacer is attached to the activated support before linkage of the ligand.

Methods for chemical activation of various supports and coupling of ligands for use in a number of bioaffinity systems have been extensively reviewed (69-71). Agarose itself can be

activated in many ways. The most commonly used procedure is its activation by cyanogen bromide (72).

The use of long chain bifunctional reagents such as bisoxiranes or divinylsulfone avoids the necessity of introducing a spacer molecule between the ligand and support (73).

1.3 Immobilised enzymes

1.3.1 General aspects of immobilised enzymes

A general definition for the immobilisation of an enzyme is the physical entrapment or localisation of the enzyme molecule during a continuous catalytic process. It has been recommended that the term 'immobilised enzyme' should be used to describe all enzyme preparations in which the enzyme is constrained one way or another within the limited confines of the supporting polymer (74).

Great attention has been focused on the isolation of pure and soluble enzymes that there is a tendency to forget that most enzymes *in vivo* are either attached to membranes or embedded in a viscous environment. Immobilised enzymes offer the opportunity of studying enzyme systems which may closely resemble enzyme systems *in vivo*. A clearer and truer picture of enzyme structure and catalysis could be then visualised. Studies in these directions were discussed by Brown (75) and Mosbach (76).

However, studies of immobilised enzymes may have been inspired by the possibilities of using these enzyme derivatives in many practical applications. Immobilised enzymes are being used in the field of medicine, clinical analysis, food and the pharmaceutical industries, and a large proportion of research is channeled towards these purposes (77-79).

There are many methods developed for enzyme immobilisation, and excellent reviews have been written on this topic (65,78,80).

Enzymes can be directly adsorped onto insoluble support as illustrated by Nelson and Griffin (81) who used charcoal and aluminium hydroxide for adsorption of β-fructofuranosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26). This technique has limited use as the enzymes are liable to desorption by changes in pH, ionic strength and temperature of assay systems (82). Alternative methods such as gel entrapment as adopted by Hick and Updike (83) for immobilisation of D-amino acid oxidase (D-amino acid : oxygen oxidoreductase, EC 1.4.3.3) in polyacrylamide gel, and microencapsulation technique as used by Chang and Poznansky (84) for encapsulation of catalase in collodoin membrane, generally require no chemical modification of the enzymes. These methods results in fairly high enzyme activity retention but are subject to enzyme leakage during usage.

Probably the best method for enzyme immobilisation is by covalent attachment of enzymes to insoluble supports (65). This technique involves chemically coupling the enzyme molecule to a support through its free amino, carboxyl, hydroxyl, sulphydryl, phenol or imidazole groups. As there are multiple duplications of these groups in each enzyme molecule, there may be partial loss of activity on immobilisation, assuming some of the groups used in the coupling are catalytically essential.

There appears to be no way of predicting the activity of an enzyme on immobilisation. Retention of activity depends on many factors and are peculiar for each particular enzyme. Each enzyme must be treated in isolation and immobilised in a manner suitable to that enzyme and its proposed use.

When an enzyme is immobilised to an insoluble support, or entrapped within a lattice structure, restrictions are imposed on the enzyme molecules often leading to changes in its physical and conformational properties. There is generally a lowering of activity when an enzyme is immobilised. The decrease in activity may be due to the destruction of some of the enzyme molecules or partial reduction of all bound enzymes or more likely the combination of both. Loss in activity may be attributed to the use of certain side chains from the active sites during the coupling process. Certain side chains used in the covalent linkage may be essential for the tertiary conformation of the enzyme molecule. The tertiary conformation may also be disrupted by interaction with hydrophobic supports and strains from covalent linkage. Conversely there can be enhanced stability to denaturants, proteolytic and thermal activation (65,77,80).

The effect of ionic support may cause a shift in the pH profile of the enzymes. Enzyme bound to positively charged matrix was shown to have its pH profile shifted to the left (acidic), whereas enzyme bound to negatively charged support has its pH profile shifted to the right (alkaline) (80). These shifts may be due to the microenvironmental effect where there may be greater or lesser 'local concentration' of hydrogen ions then in the bulk solution when the matrix is negatively or positively charged respectively.

Diffusional effect is also prominent in immobilised enzyme systems. The enzyme particles may be covered by an unstirred

layer which affects the diffusion of substrates or products in the vicinity of the immobilised enzyme molecule (85,86).

1.3.2 Immobilisation of enzymes onto nylon, and their use in automated analysis

Nylon is an unbranched polymer containing secondary amide, typically synthesised by condensing a *bis* aliphatic amine with a *bis* aliphatic carboxylic acid. Nylon 6 is the polymerisation product of caprolactam, and has a single repeating 6-carbon unit of five methylene and an amide group. Nylon 66 on the other hand is the polycondensation product of adipic acid with hexamethylene diamine, giving a polymer of alternate 6-carbon unit of four and six methylene units respectively.

Nylon polymer makes ideal support matrices for enzyme immobilisation. It is mechanically strong, resistant to microbial attack, and it is available at moderate cost in different forms of mesh, powder, membrane and tube.

High molecular weight nylon polymer has few free amino or carboxyl groups, but the secondary amide groups are potentially reactive. Secondary amide groups can be cleaved hydrolytically under acidic condition to yield free amine or carboxylic groups (87), or non-hydrolytically with an amine such as N,N-dimethyl-1,3-propane diamine under non-aqueous condition (88).

Hydrolysis may cause the loss of structural integrity of the nylon polymer. Goldstein et al. (89) generated reactive centres by N-substitution of secondary amide involving mild

acid hydrolysis followed by a four components condensation reaction between amine, carboxyl, aldehyde and isocyanide which resulted in the restoration of intact nylon backbone.

Campbell et al. (90) O-alkylated nylon with dimethyl sulphate at 100° for 3 min. However this method is rather harsh and difficult to control. Milder conditions were described by Morris et al. (91) using triethyloxonium salt, a procedure optimised by Noy (92).

Introduction of a bifunctional amines or hydrazides between the polymer and the enzyme molecule was found to produce higher activity immobilised enzymes (90).

The coupling of enzymes to the activated nylon tube can be achieved by the bisimidate or more frequently by the glutaraldehyde reaction.

The coupling of enzymes through bisimidates is brought about by the well characterised reactions of free amino groups with imidates to form amidines (93).

There is some doubt about the exact mechanism of glutaraldehyde action. It is found to be very reactive to the N-terminal amino acid, the sulphydryl group of cysteine and there is partial reactivity with the imidazole group of histidine (94). Studies on glutaraldehyde solution shows that it comprises primarily cyclic forms of hemihydrate (71%) whereas there is only a minor

fraction of the linear form (4%) at 23° (95,96). Korn et al. (97) attributed the reactivity of glutaraldehyde to the free linear form, but Whipple and Ruta (96) proposed that the prominent role was contributed by the hemiacetal form.

Monsan et al. (98) using NMR and IR spectroscopy found a polymerised form of glutaraldehyde as the stable form of the compound in alkaline solution. This polyglutaraldehyde is similar in structure to the one porposed by Richard and Knowles (99), but these groups of workers differ in their interpretation of the reaction mechanism. The latter hypothesised a Michael-type of reaction, whereas the former proposed a mechanism in which the aldehyde group appeared to be more reactive than the ethylenic group in the polymer (Fig. 1.3.2.1).

The advantages offered by using immobilised enzymes in analysis was discussed by Hornby and Noy (100). Immobilised enzymes can be reused after each analysis. In the batch mode application, the immobilised enzymes can be retrieved either by filtration or centrifugation, or in the flow through mode, substrates or samples of analyte can be perfused continuously through the immobilised enzyme reactors. Many immobilised enzyme derivatives possess greater operational and storage stability, important properties for analytical reagents. These two factors combine to a third factor, the 'convenience' factor in the use of these enzyme derivatives.

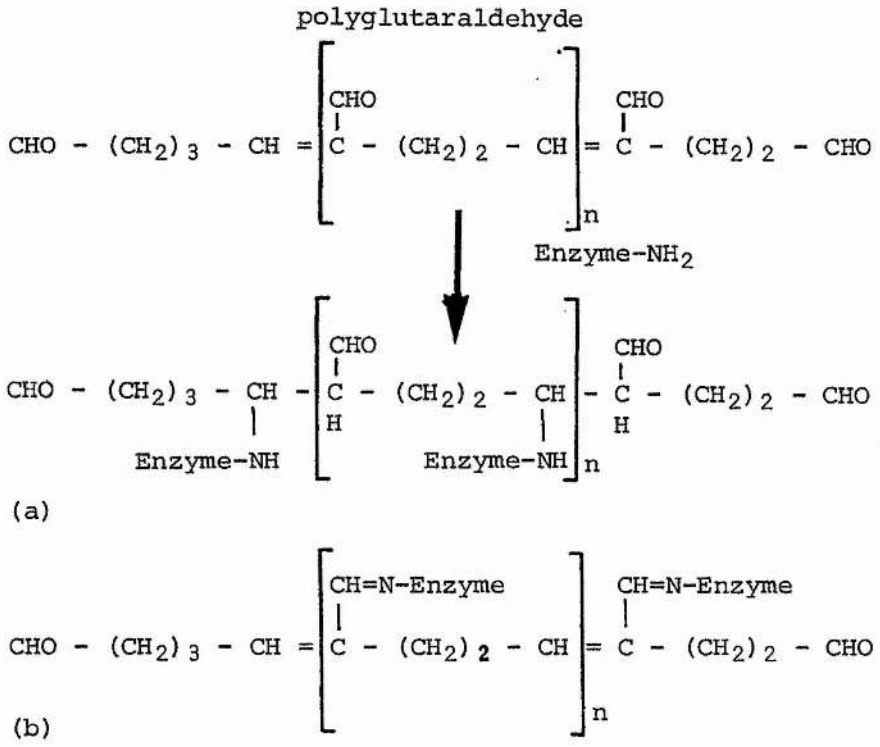


Fig. 1.3.2.1 Reactions of glutaraldehyde with the free amino acid group of an enzyme.

Application of immobilised enzymes in analysis have been reviewed on several occasions (101,102).

Novel analytical hardware systems have been developed, especially to accomodate and capitalise on the properties of the immobilised enzymes. Such an approach is illustrated by the development of enzyme electrodes (103), enzyme thermistors (104) and continuous flow systems (105).

The other approach is the incorporation of immobilised enzymes onto existing analytical hardware (105). Several types of immobilised enzyme derivatives have been used in continuous flow analysers of the type developed by the Technicon Instrument Company. Immobilised enzymes reactors are incorporated into the continuous flow system, and discreet samples are automatically analysed and the results recorded.

Glucose oxidase (β-D-glucose : oxygen oxidoreductase, EC 1.1.3.4) and urease (urea amidohydrolase, EC 3.5.1.5) have been immobilised by cross-linking in pores of nylon membrane, for use in the automated analysis of glucose and urea (87). Small packed beds of nylon powder covalently bound with urease and uricase have been developed for use in the automated determination of urea and uric acid (106).

Open tubular enzyme reactors are found to be most suitable for this kind of flow system. Glucose oxidase has been immobilised to polystyrene tube (107), and aldehyde, alcohol and glucose dehydrogenases (glucose dehydrogenase; β-D-glucose : NAD(P) oxidoreductase, EC 1.1.1.47) have been covalently immobilised

onto nylon tube and incorporated into automated analysers in the analysis of their respective substrates (92).

In this work, a new procedure for purifying uricase from porcine liver is presented, utilising bioaffinity chromatography techniques. Nylon tube is activated directly by glutaraldehyde and purified uricase is immobilised to the activated nylon tube for use in the automated analysis of uric acid in serum.

2. MATERIALS

The chemicals listed below are used in the relevant sections of this thesis. Abbreviated names of suppliers are included. Unless otherwise specified, the chemicals listed are of laboratory grade.

Substrates and inhibitors

Cyanuric acid	Aldrich
β-D-glucose	Sigma
Potassium oxonate	Aldrich
Potassium urate	Sigma
Uric acid	B.D.H.
Xanthine	Sigma

Enzymes and proteins

Bovine serum albumin	Sigma
Glucose oxidase, fungal origin	Boehringer
Human serum albumin	Sigma
Myoglobulin	Sigma
Peroxidase, Type 11, from horse radish	Sigma
Uricase, Type IV, from *Candida utilis*	Sigma

Chromatographic components

DEAE-cellulose (DE52)	Whatman
Sephadex G25 (fine)	Pharmacia
Sephadex G200 (superfine)	Pharmacia

Electrophoresis components and staining agents

Acrylamide (electrophoresis grade)	B.D.H.
3-Amino-9-ethylcarbazole	Aldrich
Coomassie Blue R250	B.D.H.
N,N-dimethyl formamide	B.D.H.
2-Mercaptoethanol	B.D.H.
N,N-methylene bisacrylamide (electrophoresis grade)	B.D.H.
Riboflavin	Sigma
Sodium dodecyl sulphate (electrophoresis grade)	B.D.H.
5,6,7,8-tetrahydro-1-napthylamine	Aldrich
N,N,N',N'-tetramethylethylenediamine	B.D.H.

Support and coupling agents

Boron trifluoride diethyl etherate	Aldrich
1,4-Butanediol diglycidyl ether	Aldrich
Dimethyl suberimidate	Sigma
Epichlorohydrin	Aldrich
Glutaraldehyde, 25%(w/v)	B.D.H.
Nylon 6 tube (1mm, internal diameter)	Portex
Sepharose 4B	Pharmacia

Miscellaneous

Adipic hydrazide	Cambrian
4-Aminophenazone	B.D.H.
L-Ascorbic acid	Fison
L-cysteine	Sigma

Dialysis tubing	Sci. Instr.
Diaminoethane	B.D.G.
3,5-Dichloro-2-hydroxybenzene sulphonyl chloride	Aldrich
Folin-Ciocalteau reagent	Sigma
L-Glutathione	Sigma
Kjeldahl(mercury) tablets	B.D.H.
Polyethyleneimine (polymin P)(50%,w/v)	B.D.H.)
Potassium alumina silicate (molecular seive, type 3A)	B.D.H.
Sulphuric acid, nitrogen free	B.D.H.
Wellcomtrol serums	Wellcome

Solvents

Methanol (B.D.H.) was distilled and dried over molecular sieve. Dichloromethane (B.D.H.) and diethyl ether (B.D.H.) were distilled and stored over calcium hydride (B.D.H.).

All other reagents used were of reagent grade.

3. GENERAL METHODS

3.1 Assay of soluble enzyme activity

3.1.1 Uricase

Uricase activity was assayed by the rate of oxygen consumption, as shown in the reaction below,

$$\text{Uric Acid} + O_2 + H_2O \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

A unit of uricase activity is defined as one µmole of oxygen consumed min^{-1}.

Specific activity is defined as the units of uricase mg $protein^{-1}$.

Reagents

1. 0.1M Borate buffer, pH 8.6.

2. Substrate, 4.46mM urate.
   75 mg uric acid and 60 mg lithium carbonate were dissolved in warm distilled water (60°). After cooling the solution was made up to 100 ml.

3. Uricase solution.

Procedure

Buffer (5 ml) and enzyme (10-100µl) were equilibrated in the reaction chamber at 25°. The reaction was initiated by the

addition of 100µl substrate.

Oxygen was monitored by a Yellow Spring Model 53 Oxygen Monitor. This unit was attached to a Devices DC6 amplifier and a Kipp & Zonen DB8 recorder.

Saturated oxygen in water was taken as $230\mu l.\ell^{-1}$. To calibrate the instrument, saturated sodium dithionite was used for complete oxygen consumption.

3.1.2 Glucose oxidase (GOD)

Glucose oxidase activity was determined by the rate of oxygen consumption, as shown below,

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{GOD} \beta\text{-D-gluconolactone} + H_2O_2$$

A unit of GOD activity is defined as one µmole of oxygen consumed min^{-1}.

Specific activity is defined as unit of activity mg $protein^{-1}$.

Reagents

1. 0.1M Phosphate buffer pH 7.0.

2. Substrate - 0.55M glucose.
   10 g D-glucose was dissolved in phosphate buffer and made up to 100 ml. The solution was allowed to mutarotate for at least 24h. before use.

Procedure

4.0 ml of buffer and 1.0 ml of substrate were equilibrated in the reaction chamber at 25°. The reaction was initiated by the addition of 20-100µl GOD.

Oxygen consumption was monitored by the oxygen electrode as described in section 3.1.1.

3.2 Methods for determination of proteins

3.2.1 Protein estimation by the Micro-Biuret method

This method developed by Itzhaki and Gill (108) was used for estimation of protein solution in the range of 0.2-2.0 mg. ml^{-1}.

Reagents

1. Solution A: 0.21%(w/v) $CuSO_4.5H_2O$ in 30%(w/v) NaOH.
2. Solution B: 30%(w/v) NaOH.
3. Protein standards. Bovine serum albumin.

Procedure

A_1 - 2 ml distilled water + 1 ml solution A.

A_2 - 2 ml protein solution + 1 ml solution A.

B_1 - 2 ml distilled water + 1 ml solution B.

B_2 - 2 ml protein solution + 1 ml solution B.

The solutions were thoroughly mixed and the absorbance at 310nm were read after 5 min. Solution A_2 was read against solution A_1 and solution B_2 was read against B_1. The difference in the two readings, Absorbance A - Absorbance B was taken as the net absorbance.

Standard protein solutions in the range of 0.2-2.0 mg. ml^{-1} were similarly treated. A graph of net absorbance against protein concentration was plotted as shown in Fig. 3.2.1.1.

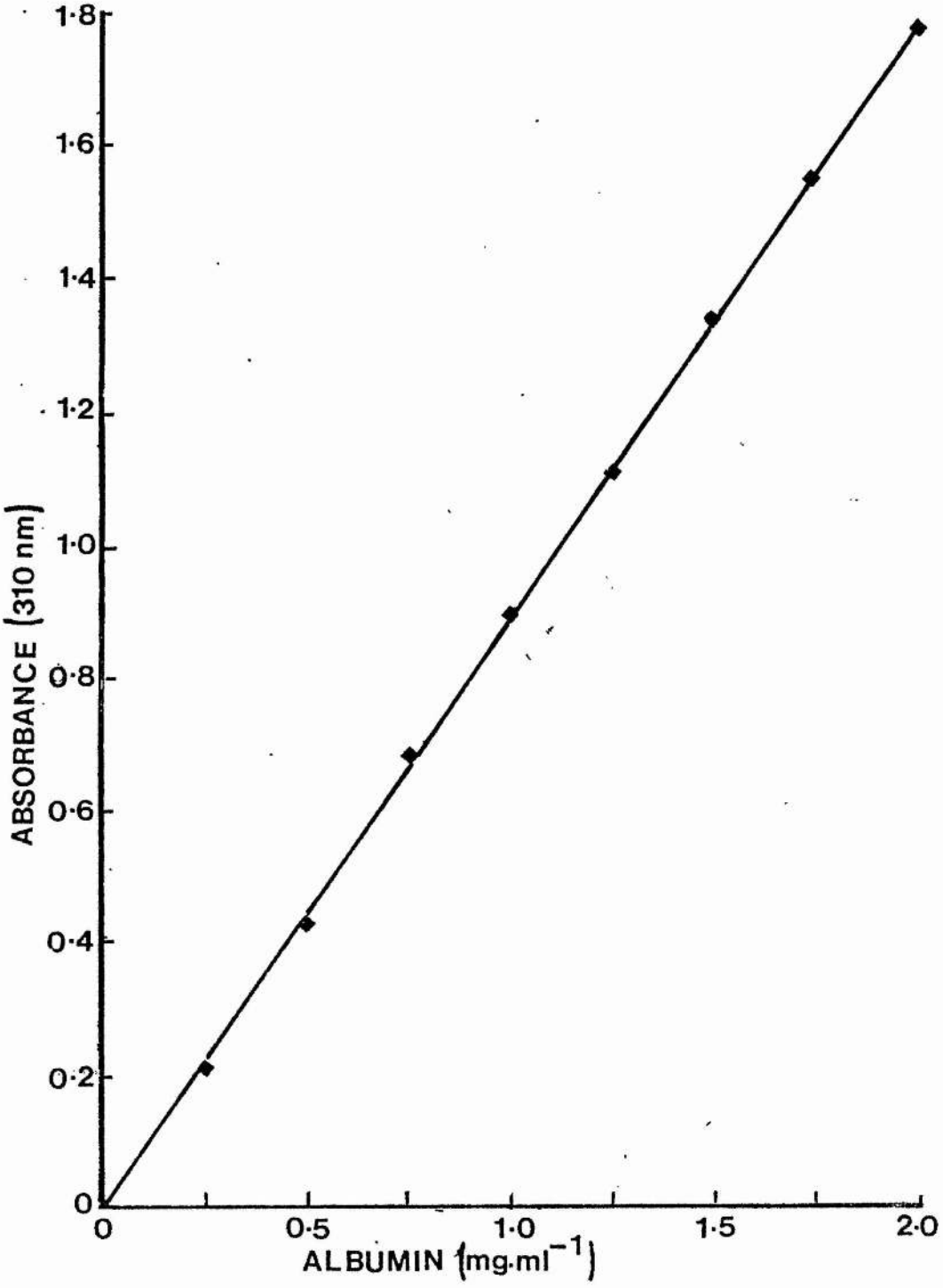


Fig. 3.2.1.1 Standard curve for the determination of proteins (section 3.2.1).

3.2.2 Protein estimation by the Folin-Lowry method

This method was developed by Lowry et al. (109) involving the reaction of copper in alkaline solution and the reduction of Folin-Ciocalteau reagent. This method was used in estimating low concentration of protein solutions.

Reagents

1. Solution A: 2%(w/v) sodium carbonate in 0.1M NaOH.
2. Solution B: 0.5%(w/v) $CuSO_4.5H_2O$ in 1%(w/v) sodium tartrate.
3. Solution C: 50 ml of solution A was mixed with 1 ml solution B.
4. Solution D: Folin-Ciocalteau reagent, diluted to 1M with distilled water.
5. Protein standards. Bovine serum albumin.

Procedure

0.5 ml protein solution was mixed with 3 ml solution C and allowed to stand at room temperature for 15 min. An aliquot of solution D (0.3 ml) was then added to the mixture and immediately shaken and allowed to react for a further 30 min. The absorbance was read at 500nm and 750nm against a blank containing no protein.

A calibration plot was prepared by assaying standard solutions in the range 25-200$\mu g.ml^{-1}$. Fig. 3.2.2.1 shows the plot obtained.

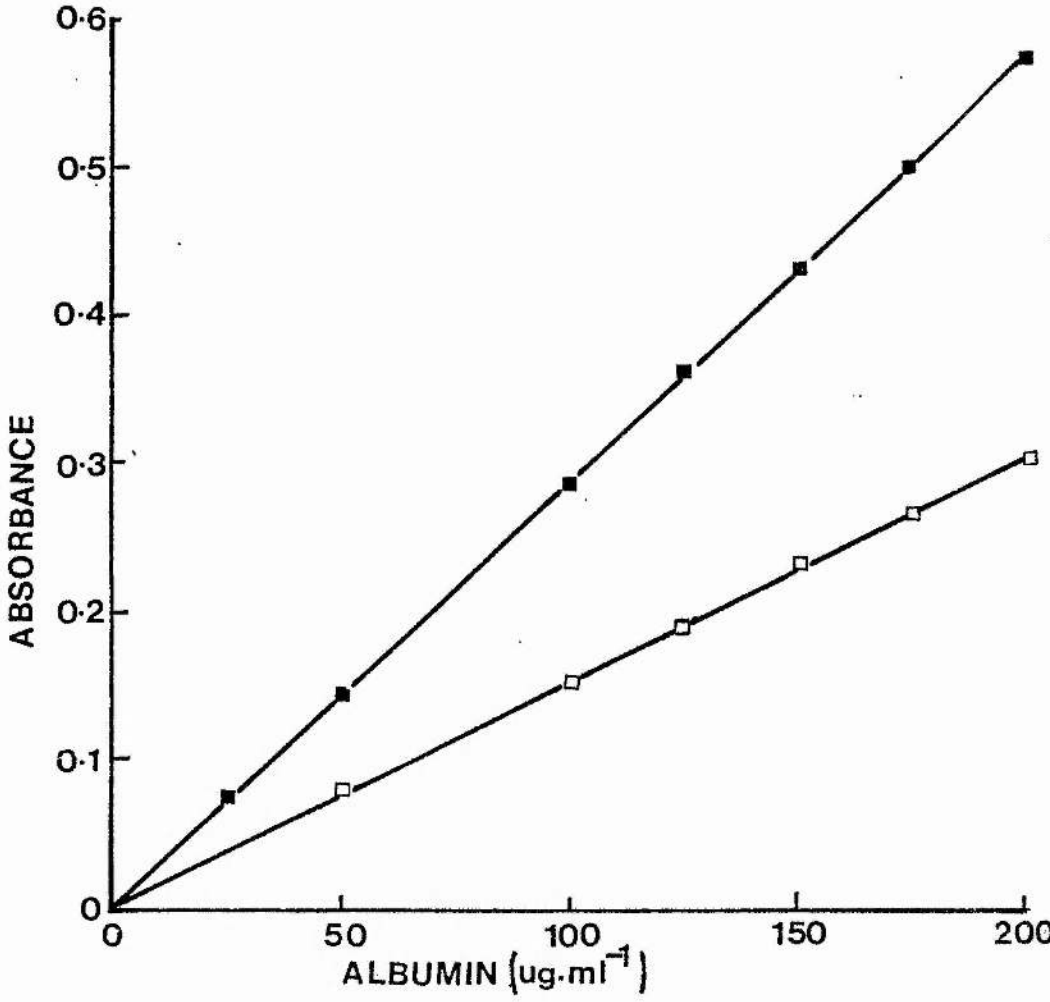


Fig. 3.2.2.1 Standard curves for the determination of proteins (section 3.2.2). (□) indicates absorbance at 500nm, and (■) indicates absorbance at 750nm.

3.3 Buffers

Buffers were prepared by weighing appropriate amount of acid or base and diluting in water and titrating the solution to the required pH.

Typically 0.1M phosphate buffer pH 7.0 was prepared by weighing 13.6g KH_2PO_4 and dissolving it in about 800 ml water. The solution was titrated to pH 7.0 by 2M NaOH and made up to 1ℓ.

Other buffers used are listed below. Appropriate amount of the first component was weighed and dissolved in water and titrated with the second component.

1. Boric acid - NaOH.

2. Glycine - NaOH.

3. TRIS - HCl.

The pH of all buffers was checked before use with the Radiometer pH meter (Copenhagen). The instrument was calibrated daily with reference buffers.

3.4 Dialysis

Dialysis tubing was prepared by boiling in 10mM EDTA for 20 min and then washed thoroughly in distilled water. The tubing was washed with the dialysis buffer before use. Dialysis was carried out at 4°.

Dialysis tubing was stored in 0.1%(v/v) sodium azide at 4°.

3.5 Centrifugation

Low speed centrifugation (less than 2000 x *g*) was carried out on the MSE Mistral 6L centrifuge using appropriate rotors. Centrifugation requiring 5000 x *g* or greater were carried out on the MSE High Speed 18.

All centrifugations were carried out at 4° unless otherwise specified.

4. PURIFICATION OF URICASE FROM PORCINE LIVER

4.1 Methods

4.1.1 Preparation of liver homogenate

The use of alkaline buffer to extract uricase from microbodies was utilised by Yokota (43). Studies on the effect of detergent, ionic strength and pH for the extraction and solubilisation of uricase have been carried out (110,111). Borate ions and EDTA appear to confer a stabilising effect on uricase (112).

Reagents

1. Porcine liver. It was acquired immediately after slaughter and cooled in ice. It was either used on the day or stored frozen at -20°.

2. Buffers

   a. 0.1M borate, 1mM EDTA, pH 10.

   b. 0.1M borate, 1mM EDTA, pH 11.

   c. 0.1M borate, 1mM EDTA, 1.5%(v/v) Triton X100, pH 10.

   d. 0.1M borate, 1mM EDTA, 3%(v/v) n-butanol, pH 10.

   e. 0.1M borate, 1mM EDTA, 3%(v/v) n-butanol, 1.5%(v/v) Triton X100, pH 10.

Procedure

Porcine liver was cut into small pieces, and as much tendon and fatty tissues as possible were removed. To every 100 g. liver,

250 ml buffer was added. The mixture was homogenised in a Waring blender for 1 min and the homogenate stirred for 2h at 4°.

The homogenate was diluted with 2 volumes of buffer, and stirred for a further 30 min. The homogenate was filtered through double layer muslin cloth.

For the purpose of monitoring the protein content, the homogenate was centrifuged at 5000 x *g* for 30 min and the supernatant assayed for protein (section 3.2.1).

4.1.2 Heat treatment of homogenate

This is a common procedure adopted mainly for removal of some proteins, particularly enzymes of low thermal stability.

Procedure

Liver homogenate was placed in a conical flask, and the flask was immersed in an 80° bath. The homogenate was swirled around in the flask until the temperature of the homogenate reached 60°. The flask was immediately removed from the bath and stood at room temperature (ca. 20°) for 5 min. The temperature of the homogenate should stay in the range of 55-60° within this period. The temperature of the homogenate was then quickly brought down to under 10° by immersing the flask in an ice bath.

To assay for protein the treated homogenate was centrifuged at 5000 x *g* for 30 min and the supernatant assayed for protein by the micro-Biuret method (section 3.1.2).

4.1.3 n-Butanol separation

This is a two-phase separation procedure as discussed by Morton (113) and is regarded as a general purification procedure.

Procedure

n-Butanol was chilled at -20° before use. Cold n-Butanol was slowly added to heat treated homogenate with stirring. An over-head stirrer was necessary as the suspension got heavier with precipitation with addition of alcohol. The suspension was stirred for 15 min after complete addition of the n-butanol (1:1,v/v).

The suspension was centrifuged at 2000 x *g* for 60 min. The suspension was separated into three layers, an upper butanol layer, a middle butanol saturated residue and an aqueous layer containing uricase activity. The two upper layers were discarded.

4.1.4 Ammonium sulphate precipitation

This is a general procedure adopted for concentration of the protein extract with some measure of purification.

Procedure

Ammonium sulphate crystals were finely grounded and dissolved in a protein extract obtained as described in section 4.1.3. Small fractions of ammonium sulphate were added at a time with constant stirring allowing the salt to dissolve and thus minimising high local concentration of salt in the solution. The solution was kept cool in an ice bath.

On complete addition of ammonium sulphate, the suspension was allowed to stand for at least 1h. The suspension was centrifuged at 5000 x g for 30 min. The precipitate was separated at the top of the centrifuged tube and harvested. The supernatant was discarded.

The precipitates were mixed with 0.1M borate, 1mM EDTA pH 10.0 and stirred for 30 min. The suspension was centrifuged at 5000 x g for 30 min and the supernatant collected. The residue was re-extracted if necessary and the supernatant collected after centrifugation.

The supernatant which contained uricase activity was stored at -20°, when not required for immediate use.

4.1.5 Ion exchange chromatography on DEAE-Cellulose

As this is one of the most regularly used purification techniques, its applicability in the purification of uricase was studied.

Procedure

DEAE-cellulose was prepared according to the instruction of the manufacturer (Improved Technique with Advanced Ion Exchange Cellulose-Whatman).

The ion-exchanger was packed in a column (1 x 12 cm) to a height of 10 cm. The DEAE-cellulose was equilibrated with 10mM borate, pH 8.5.

Uricase extract from ammonium sulphate precipitation (section 4.1.4) was dialysed in equilibrating buffer for 16h. The dialysed sample was passed through the column at the rate of 0.5 $ml.min^{-1}$. The ion-exchanger was washed with the equilibrating buffer and a salt gradient of ammonium sulphate (0-0.25M, 160 ml) was passed through the column.

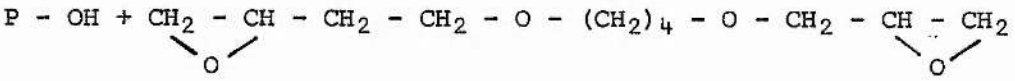
Fractions were assayed for uricase and protein was monitored at 280nm.

4.1.6 Bioaffinity chromatography

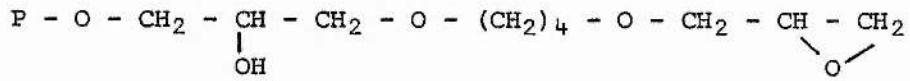
4.1.6.1 Preparation of affinity support

a. Activation of sepharose 4B

The method used was developed by Sunberg and Porath (114). The reaction is shown below,



Sepharose 1,4-butanediol diglycidyl ether.



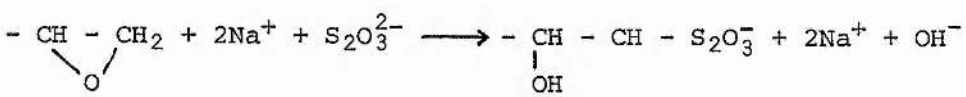
Procedure

50 g suction-dried Sepharose 4B was washed with 1ℓ of distilled water. The gel was mixed with 50 ml 0.6M NaOH containing 100 mg sodium borohydride and 50 ml of 1,4-butanediol diglycidyl ether. The mixture was stirred for 8h at 25°.

The activated gel was washed with at least 1ℓ distilled water to remove excess 1,4-butanediol diglycidyl ether. The activated gel was used immediately for ligand coupling.

b. Determination of oxirane groups attached to Sepharose 4B

Oxirane group reacts with sodium thiosulphate to produce sodium hydroxide as shown in the reaction below.



oxirane

Procedure

5 g. suction-dried activated Sepharose 4B was added to 15 ml 1.3M sodium thiosulphate. The release of hydroxyl groups was monitored by titration against 0.1M HCl. The amount of oxirane group $g.^{-1}$ of gel was calculated by µmole of HCl required to keep the solution at neutrality.

i.e. the amount of oxirane groups attached $g.^{-1}$ of Sepharose 4B,

$$= \frac{\text{volume of HCl} \times 0.1M}{5} \text{ µmole}$$

c. Coupling of ligands to the activated Sepharose 4B

The chemistry of binding of ligands to the activated gel is assumed to be similar to the activation of Sepharose 4B.

Reagents

1. Uric acid

   0.25%, 0.5%, 1.0%(w/v) in water pH 12.

2. Xanthine

   0.5, 1.0%(w/v) in water pH 12.

3. Cyanuric acid

   0.5, 1.0%(w/v) in water pH 12.

4. Oxonic acid, potassium salt

   1.0%(w/v) in water pH 12.

Procedure

10 g of activated Sepharose 4B was mixed with 50 ml of ligand solution and the mixture stirred for 20 h at 25°.

The gel was washed with 0.1M NaOH and distilled water. Appearance of urate in the wash was monitored at 293nm. Oxonate was monitored at 314nm, xanthine at 277nm and cyanurate at 214nm. The gel was washed until no ligand was detected in the wash.

Unreacted oxirane group on the gel was removed by incubating the gel with 1M N-ethanolamine overnight at 25°. The gel was washed with distilled water and stored in 0.1M borate pH 9.0, containing 0.1%(w/v) sodium azide at 4°.

d. Determination of ligands coupled to Sepharose 4B

The amount of ligand attached to the gel was estimated by the determination of nitrogen content by the modified Kjeldahl method.

Reagents

1. Kjeldahl catalyst tablet. Each tablet contains 1 g sodium sulphate and 0.1 g mercury.

2. Alkaline solution. 60%(w/v) NaOH containing 5%(w/v) sodium thiosulphate.

3. Nitrogen free concentrated sulphuric acid.

4. Borate buffer.

10 g boric acid was dissolved in about 700 ml distilled water and 200 ml absolute ethanol. 10 ml mixed indicator was added to the solution and it was adjusted to the required end point (grey) with 0.1M NaOH. The volume was made up to 1ℓ with distilled water.

5. Mixed indicator.

33 mg Bromocresol green and 66 mg methyl red indicator were dissolved and made up to 100 ml in absolute ethanol.

6. HCl, 0.01M.

Procedure

1 and 2 g suction dried Sepharose-ligand (as prepared in section 4.1.4.1c, obtained before incubation with N-ethanolamine) were dried at 100° over-night. Each sample was placed in a Kjeldahl flask and the digestion comprising half a tablet mercury catalyst, 2 ml nitrogen free concentrated sulphuric acid and 2 anti-bump granules were added. The sample was digested for 3h and then allowed to cool at room temperature.

The mixture was transferred to the distillation apparatus, washed down twice with 3 ml distilled water. Ammonia was released from the mixture by adding 10 ml alkaline solution with steam distillation. The distillate was collected in a flask containing 5 ml borate buffer to a total volume of 50 ml.

The distillate was titrated against 0.1M HCl.

Samples of uric acid (1-2 mg) were similarly treated and the recovery of nitrogen was estimated. Fig. 4.1.6.1.1. shows a plot of nitrogen recovery against digestion time.

4.1.6.2 Use of affinity support for uricase purification.

a. Capacity of affinity support

2 g suction-dried Sepharose-ligand was packed in a glass column (1 x 7 cm) and equilibrated with 0.1M borate containing 1mM EDTA pH 9.0.

A uricase sample as prepared in section 4.1.4 was dialysed in the equilibrating buffer for 16h. The sample was perfused through the affinity bed at a flow rate of 0.4 $ml.min^{-1}$.

The column of affinity support was maintained at 4°. The column effluent was monitored for uricase activity and protein content. The perfusion was terminated when the output sample activity was about 50% of the input activity.

b. Elution of uricase from affinity support

Eluants were passed through the affinity support packed in the column at the same rate as the adsorption procedure. Three different eluants were considered for elution of uricase from the affinity support.

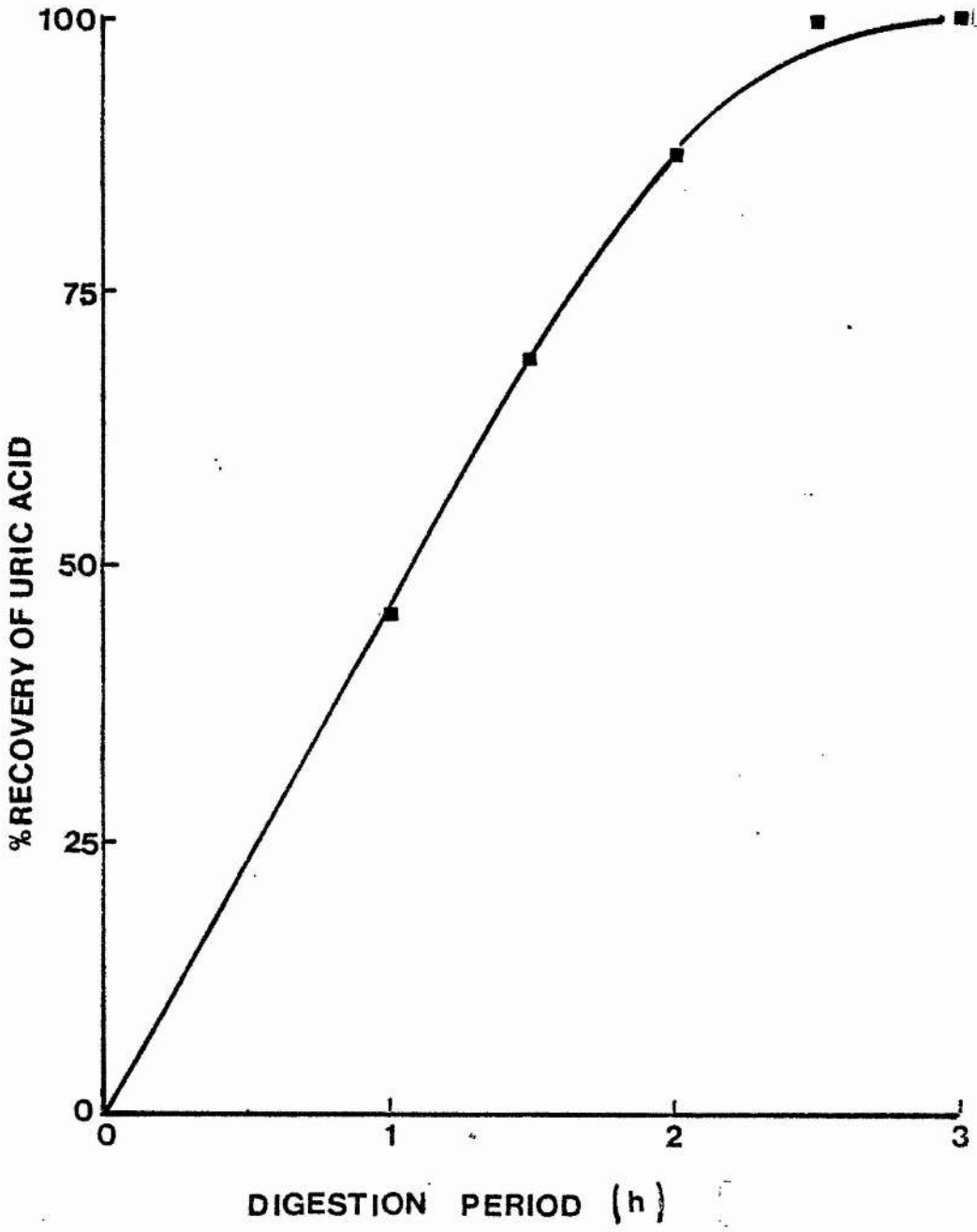


Fig. 4.1.6.1.1 Recovery of uric acid with period of digestion. Nitrogen was determined by the Kjeldahl method (section 4.1.6.1d).

1. Uric acid in 0.1M borate buffer pH 9.0.

2. Xanthine in 0.1M borate buffer pH 9.0.

3. Potassium oxonate in 0.1M borate pH 9.0.

4.1.7 Separation of inhibitors from uricase

Eluates from the affinity chromatography procedure were passed through Sephadex G25, to obtain the purified uricase.

Procedure

Sephadex G25 was prepared for use according to instruction of the manufacturer (Sephadex, Gel Filtration in Theory and Practice-Pharmacia). 30 g dry weight of Sephadex G25 was allowed to swell in water for 3h at room temperature. The gel was packed onto a column (1.6 x 70 cm) up to a height of 60 cm. The columm was maintained at 4°.

The gel equilibrated with 0.1M borate, pH 9.0.
A 4-10 ml sample was applied to the column and the gel perfused with equilibrating buffer at the rate of 12 $ml.min^{-1}$.

4 ml fractions of eluate were collected. Protein was monitored at 280nm, and active fractions were assayed for protein by the Folin-Lowry method (section 3.2.2).

4.1.8 Concentration of uricase solution

Uricase solution was concentrated by filtration using the Centriflo-Membrane Ultrafilter (Amicon-Membrane Cone, 224-cf-50). This membrane has a cut-off molecular weight limit of 50,000. Each membrane cone has a capacity of about 7 ml.

Filtration was achieved by centrifugation at 2000 x *g*.

4.1.9 Gel filtration on Sephadex G200

This procedure is for separation of any other proteins which may have contaminated the uricase.

Procedure

Sephadex G200 was prepared for use according to the instruction of the manufacturer (Gel Filtration, Theory and Practice-Pharmacia). 5 g dry weight of the gel was allowed to swell in water for 5h, incubated in a boiling water bath. The gel was packed in a column (1.6 x 70 cm) to a height of 60 cm, and equilibrated with 0.1M borate buffer pH 9.0. The column was maintained at 4°.

Void volume was determined by applying 2 ml (2 $mg.ml^{-1}$) Dextran Blue, and measuring the elution volume.

5 ml sample from Sephadex G25 gel filtration (section 4.1.7) was applied to the column. The protein was eluted with the equilibrating buffer at the rate of 9 $ml.min^{-1}$. All fractions collected were assayed for uricase activity and protein.

4.1.10 Polyacrylamide disc-gel electrophoresis

Two methods of polyacrylamide disc-gel electrophoresis were utilised for studying the purified uricase.

4.1.10.1 Polyacrylamide disc-gel electrophoresis by the method of Ornstein and Davis

This method followed the procedure put forward by Ornstein (115) and Davis (116). A slight modification was made in the order of gel preparation.

Reagents

Stock solutions.

A:	1M HCl	48 ml
	TRIS	36.6 g
	TEMED	0.23 ml (pH 8.9)
	Water to	100 ml
B:	1M HCl	48 ml
	TRIS	5.98 g
	TEMED	0.46 ml (pH 6.7)
	Water to	100 ml
C:	Acrylamide	28 g
	BIS	0.735 g
	Water to	100 ml
D:	Acrylamide	10 g
	BIS	2.5 g
	Water to	100 ml

E:	Riboflavin	4 mg
	Water to	100 ml
F:	Sucrose	40 g
	Water to	100 ml

The pH of the solution was adjusted by titration with 1M HCl. Acrylamide solutions were filtered to remove debris.

Working solution.

Small pore gel

This solution was made of 1 part solution A, 2 parts solution C, 1 part water and 4 parts of 0.14%(w/v) ammonium persulphate solution. The pH was adjusted to pH 8.9.

Large pore solution

This solution was made of 1 part solution B, 2 parts of solution D, 1 part solution E and 4 parts solution F. The pH was adjusted to pH 6.7.

Stock buffer

This solution was made of 6 g TRIS, 28.8 g glycine and water to a total volume of 1ℓ. The pH was adjusted with 1M HCl. The stock buffer was diluted 1:10 for use during electrophoresis.

Tracking dye

The tracking dye comprised 0.05%(w/v) Bromophenol Blue in water.

Staining solution

The solution was made of 1.25 g Coomassie 250R in 454 ml 50%(v/v) methanol in water and 46 ml glacial acetic acid.

Destaining solution

The solution was made by mixing 250 ml methanol and 75 ml glacial acetic acid and the volume made to 1ℓ with distilled water.

Preparation of gels

The separating gel was prepared first. A gel tube stoppered at one end was filled with small pore gel up to 5.5 cm height. The gel was carefully layered with water. The gel tube was placed under a fluorescent lamp (about 3 cm under the source of light) and exposed for 30 min. On polymerisation, the water layer was carefully removed and the inner wall of the tube thoroughly drained. Large pore gel (spacer gel) was layered on top of the separating gel to a height of 1 cm. The gel was layered with water and exposed to fluorescent light for 45 min.

Electrophoresis

Electrophoresis was carried out on the SHANDON ELECTROPHORESIS KIT (Shandon Co. Ltd.). 8 gel tubes could be fitted to this apparatus in a single run. 2mA current was used per gel. Electrophoresis was carried out at 4°.

Pre-electrophoresis of the gels was carried out for 2h before application of samples.

Protein samples (10-20µg) were mixed with 1 drop of glycerol and 1 drop of tracking dye, and applied to the gel for electrophoresis.

The gels were stained for 20 min with Coomassie Blue. Destaining was achieved over a period of 36h with periodic changing of the destaining solution.

4.1.10.2 SDS-polyacrylamide disc-gel electrophoresis

The method employed here is a modification of the method developed by Weber and Osborn (118). It was principally developed as a method for determination of molecular weight.

Reagents

1. Gel buffer.

   0.5 g SDS, 0.38 ml TEMED, 1.5 g TRIS and 120 g urea were dissolved in water and made up to 250 ml.

2. Acrylamide stock solution

   38.7 g acrylamide and 2.66 g BIS were dissolved in water and made up to 100 ml.

3. Chamber buffer

   3 g SDS and 6 g TRIS were dissolved in water and made up to 1ℓ, and the solution adjusted to pH 7.5.

4. Protein standard

Stock solution of proteins were as follows:

a. 5 $mg.ml^{-1}$ human serum albumin

b. 5 $mg.ml^{-1}$ bovine serum albumin

c. 5 $mg.ml^{-1}$ myoglobulin

These solutions were diluted ten times with chamber buffer before use.

Preparation of gel

5% gel was prepared by mixing 2.5 ml acrylamide stock, 7.5 ml water, 0.02g ammonium persulphate and 10 ml gel buffer. The gel mixture was poured into gel tubes and layered with water. The gel was polymerised under daylight and took about 20 min for complete polymerisation.

Treatment of samples and standards before electrophoresis

Aliquots of protein solution (10-20µg) were mixed with 10µl 2-mercaptoethanol and incubated at 37° for 2h.

Electrophoresis

Gels were subjected to 1h pre-electrophoresis before application of samples.

Samples were mixed with 1 drop of glycerol and 1 drop of tracking dye (as section 4.1.10.1). Electrophoresis was carried out at 4° for 1h, with a current of 2mA per gel applied (Apparatus was as described in section 4.1.10.1).

Staining and destaining of gels were as described in section 4.1.10.1.

After destaining the gels were scanned on the VITRATON TLD100, with the following settings:

Settings

1.	Lamp	phototungsten
2.	Slit	2.5 x 0.5
3.	Filter	570nm
4.	Level switch	-b
5.	Machine switch	-3
6.	Chart speed	7 (10 $cm.min^{-1}$)

4.1.11 Activity stain for uricase on acrylamide gel

The method was adapted from the method developed by Graham and Karnovsky (119).

Reagents

Staining medium

3 mg 3-amino-9-ethylcarbazole was dissolved in 1 ml N,N-dimethyl formamide, and 10 ml 0.05M TRIS-HCl buffer pH 8.0. A drop of 5,6,7,8-tetrahydro-1-napthylamine was added and the mixture thoroughly stirred. After 2 min standing the mixture was filtered.

3 mg potassium urate, 3 mg peroxidase and 5 mg EDTA were added to the filtrate and the mixture thoroughly mixed.

Procedure

Polyacrylamide gels obtained immediately after electrophoresis (section 4.1.10) were rinsed with 0.15M NaCl and immediately incubated in ice-cold acetone for 10 min. The gels were rinsed with 0.15M NaCl and then incubated in the staining medium at 37° for at least 3h.

Appearance of reddish brown stain on the gel would indicate the presence of uricase activity on the gel. The gel was washed in the salt solution and fixed in 4% formaldehyde for 2h.

4.1.12 Molecular weight estimation by analytical ultra-centrifugation

The molecular weight of purified uricase was determined by the method of Meniscus Depletion Sedimentation Equilibrium Analysis (119).

Procedure

Uricase sample in 0.1M borate buffer pH 9.0 was dialysed against 50mM morate pH 10.0, containing 0.1M ammonium sulphate for 24h. About 0.05 ml of the dialysed sample was required for analysis.

Instrument used

Spinco Model E Analytical Centrifuge (Beckman)

Conditions for centrifugation

Temperature - 20°

Speed - 15220 rpm

Cell - 12 mm, with double sector centre piece (two 2.5° sectors)

Optical system - Rayleigh interference optics.

Fringe displacement were measured by means of a travelling microscope (Projectorscope, PG Ltd.), readings being taken at 200 micron intervals, commencing at the meniscus along the x-scale. When a deflection of more than 10 microns occurred on the y scale between consecutive readings, the reading on the

y scale was taken at 100 micron intervals.

The partial specific volume was taken as 0.74, and the specific gravity of the solution as 1.0.

The programme of Yphantis and Roark (120) was utilised in the computation of the molecular weight values from experimental data.

4.2 Results

4.2.1 Extraction and purification of uricase from porcine liver

4.2.1.1 Preparation of crude uricase extract

The use of different buffer systems were investigated (section 4.1.1). Table 4.2.1.1.1 summarises the results obtained.

Buffer systems	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Specific Act. ($U.mg^{-1}$)
0.1M borate, pH 10	0.085	61	0.00135
0.1M borate, pH 11	0.09	65	0.00138
0.1M borate, 1.5% (w/v) Triton X100 pH 10	0.09	65	0.00138
0.1M borate, 3%(w/v) n-butanol, pH 10	0.085	63	0.00135
0.1M borate, 3%(w/v) n-butanol, 1.5%(w/v) Triton X100, pH 10	0.08	82	0.0010

Table 4.2.1.1.1 Extraction of uricase with different buffer systems. All buffer systems contained 1mM EDTA. The experiment is described in section 4.1.1.

The difference in the effeciency in terms of uricase extraction and protein solubilisation was minimal in all buffer systems studies, except in the buffer where both butanol and Triton X100 were present.

In this case there was significant increase in protein solubilisation.

The choice of 0.1M borate buffer containing 1mM EDTA and 1.5%(w/v) Triton X100, pH 10, was made in view of subsequent steps. Using this buffer the homogenate was stirred for 1.5h, after which no increase of uricase activity could be detected in the homogenate.

Dilution of this homogenate resulted in a further increase in the uricase content of the homogenate. Table 4.2.1.1.2 shows the result obtained when the homogenate was diluted in buffer containing Triton X100 (the homogenising buffer) and borate buffer containing no Triton X100.

	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	% Total activity	% Total protein
Initial homogenate	0.085	65	100	100
Dilution buffer:				
Buffer without Triton X100	0.035	27	124	124
Buffer with 1.5%(w/v) Triton X100	0.035	45	124	210

Table 4.2.1.1.2 Dilution of homogenate in the extraction of uricase. The initial homogenate was diluted with 2 volumes of the diluting buffer (section 4.1.1).

Apparently the presence of excess detergent only promoted the solubilisation of more proteins without achieving any increase in the uricase content.

The ratio of dilution was largely determined by the need to minimise the total volume of homogenate for the sake of convenience. A ratio of 2:1(v/v) buffer to homogenate was found to be minimum ratio permissible in view of the heat treatment step. Lower dilution of the homogenate resulted in coagulation of the homogenate on exposure to high temperature. In effect, liver could have been homogenised in buffer containing 0.5%(w/v) Triton X100.

Table 4.2.1.1.3 shows the typical results obtained when liver homogenate were heat treated as described in section 4.1.2.

Incubation period (min)	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Sp. Activity ($U.mg^{-1}$)
0	0.03	27.5	0.001
5	0.07	20.0	0.0035
10	0.05	17.5	0.003
20	0.03	16.25	0.0018

Table 4.2.1.1.3 Heat treatment of liver homogenate.
The experiment is as described in section 4.1.2. 100 ml volume of homogenate was used for each period of incubation.

The incubation period was found to be very critical and had to be adjusted according to the volume of homogenate used. The time required to raise and lower the temperature of the homogenate obviously depended on the volume of the homogenate and had to be considered.

The treatment of the liver homogenate to high temperature has the effect of increasing the uricase content of the homogenate (5 min exposure to heat, in this case, proved to be optimal in getting high specific activity uricase).

Separation of n-butanol was a very significant step as most of the proteins and lipids were removed from the homogenate. Table 4.2.1.1.4 summarises the results obtained in this procedure.

Procedure	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Sp. activity ($U.mg^{-1}$)
Heat-treated homogenate	0.08	20	0.004
Aqueous layer after butanol separation	0.10	8	0.125

Table 4.2.1.1.4 n-Butanol separation of heat-treated liver homogenate. The experiment was as described in section 4.1.3.

There was significant purification (x 12.5) achieved though there was a decrease in the total uricase units (a decrease of around 20%).

No uricase activity was detected in the supernatant after protein extract (as prepared in section 4.1.3) was saturated to 40% with ammonium sulphate. Fig. 4.2.1.1.1 shows the activity and protein profile obtained when precipitate resulting from various stage of ammonium sulphate precipitation was re-extracted with a fixed volume of buffer. The results show that precipitate from 40% ammonium sulphate precipitation produces high specific activity uricase extract.

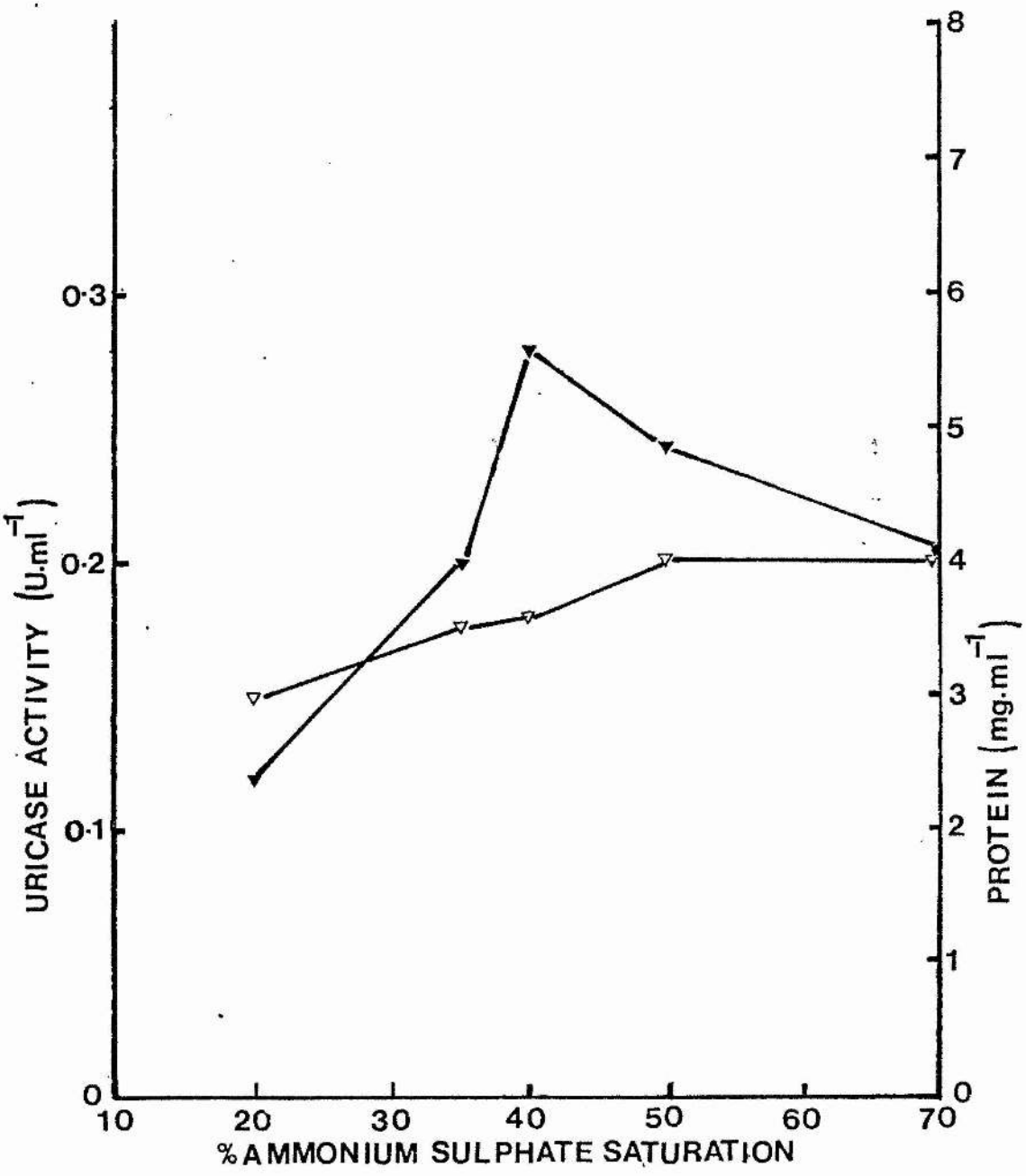


Fig. 4.2.1.1.1 Activity and protein content of uricase extract obtained from precipitates acquired by different ammonium sulphate saturation. The precipitation and re-extraction procedure was as described in section 4.1.4. Keys to the Fig.: (▼) indicates uricase activity, and (▽) indicates protein concentration.

4.2.1.2 Ion exchange chromatography

The elution profile shown in Fig. 4.2.1.2.1 represents typical results obtained by chromatographic separation on DEAE-cellulose. At pH 8.5, 95% of uricase activity in the input sample (9 U) were absorbed onto a 7 ml DEAE-cellulose bed, but only 10 to 15% of the activity were finally eluted. About 5 fold purification was achieved in the uricase recovered. Variation of pH in the absorption or eluation process did not significantly alter the elution profile shown in Fig. 4.2.1.1.1. NaCl was also utilised in the eluant without further improvement in the elution of uricase.

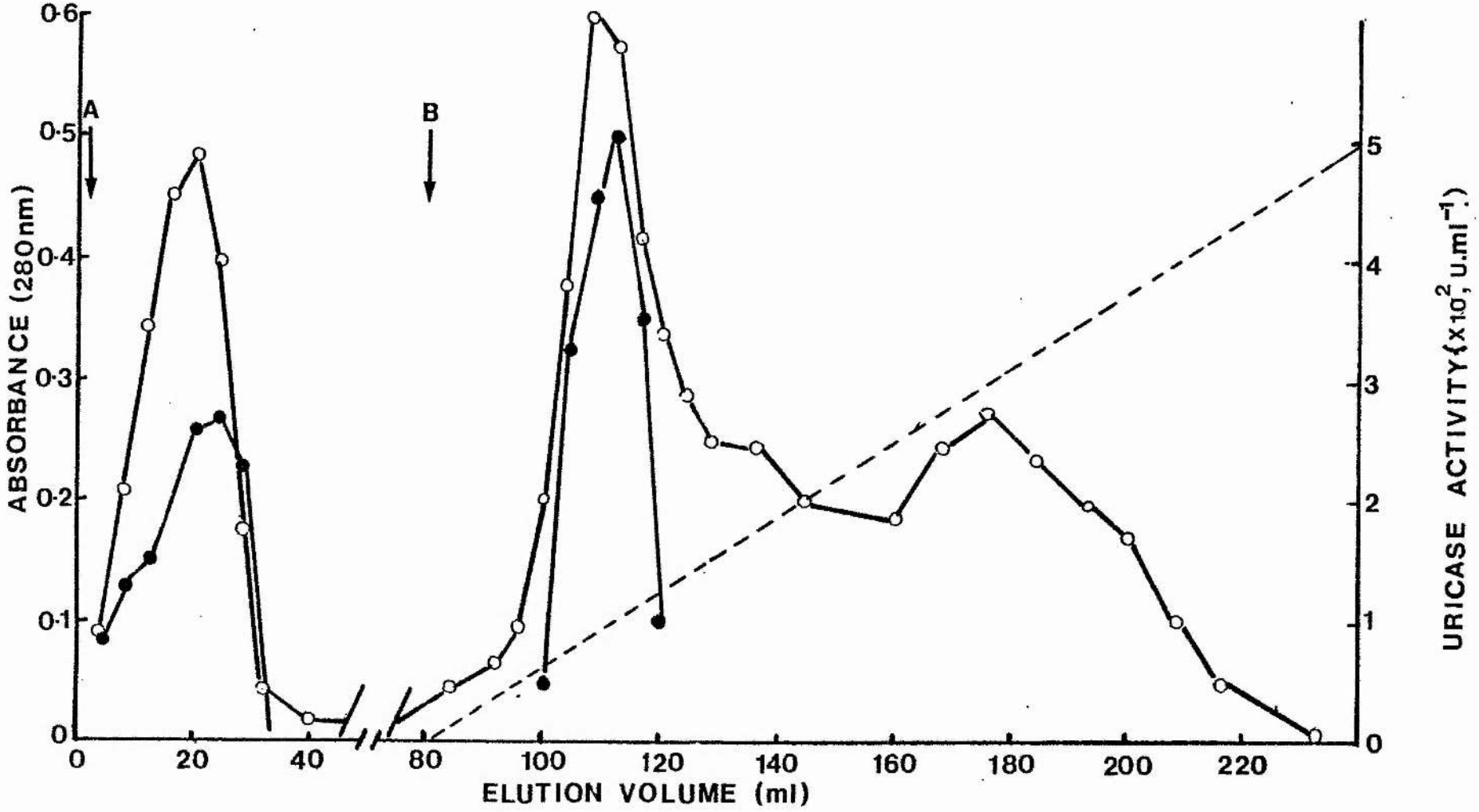
4.2.1.3 Bioaffinity chromatography of uricase

Sepharose 4B was activated as described in section 4.1.6.1a, and oxirane groups attached to the Sepharose gel was determined as described in section 4.1.6.1b. 28-30μmole of oxirane per g of suction-dried Sepharose 4B were found.

Table 4.2.1.3.1 summarises the results obtained in the coupling of ligands to activated Sepharose 4B (section 4.1.6.1c). The amounts of ligand attached to the gel were calculated by the nitrogen content of the gel, determined by the Kjeldahl method (section 4.1.6.1d).

The capacity of each Sepharose-ligand complex as an affinity adsorbent for uricase was investigated by the method described in section 4.1.6.2a, and the result is shown in Fig. 4.2.1.3.1.

Fig. 4.2.1.2.1 Elution profile of uricase separation on DEAE-cellulose. (●) indicates uricase activity, (○) indicates protein concentration and dotted line represents salt gradient (0-0.25M). Sample was applied at point A and the salt gradient applied at point B.


A
B
ABSORBANCE (280nm)
0·6
0·5
0·4
0·3
0·2
0·1
0
0
20
40
80
100
120
140
160
180
200
220
ELUTION VOLUME (ml)
URICASE ACTIVITY (x10^{-2} U.ml^{-1})
5
4
3
2
1
0

Concentration of coupling solution (100 ml⁻ 0)	µmoles ligand per g. gel
Uric acid	
1 g (0.059M)	14.25
	14.5
	14.3
0.5 g (0.03)	8.0
	8.5
0.25 g (0.015M)	5.5
	5.75
Xanthine	
1 g (0.066M)	18.5
	19.2
	19.7
0.5 g (0.033M)	11.5
	10.5
Cyanuric acid	
1 g (0.0775M)	13.6
	14.5
	13.3
0.5 g (0.039M)	9.0
	8.6
Oxonic acid	
1 g (0.064M)	5.16
	5.0
	5.3

Table 4.2.1.3.1 Amount of ligands attached to activated Sepharose 4B. Each reading is the average of 2 determinations. The different values for each concentration of ligand are for different preparations.

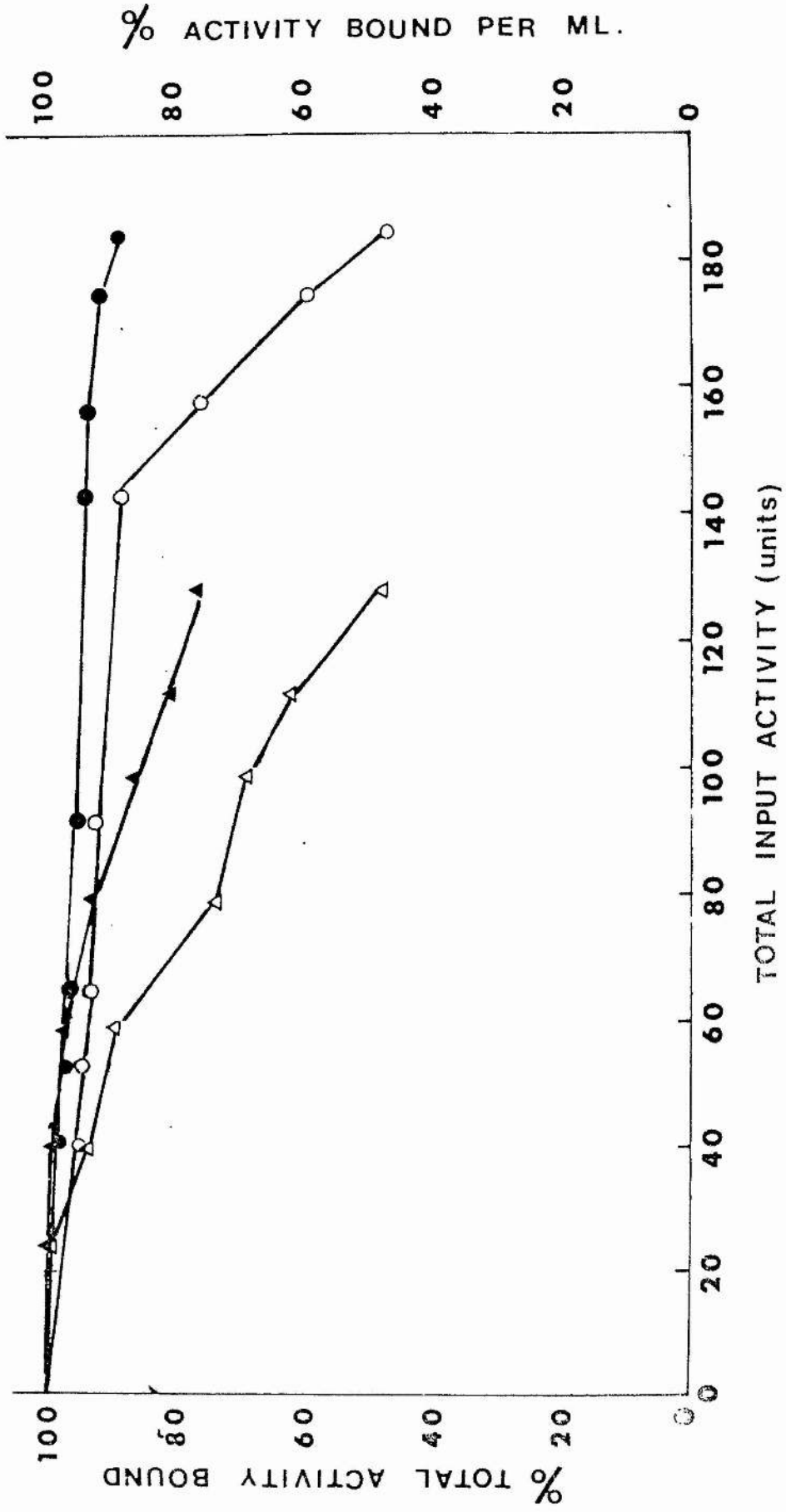
Although there was more xanthine bound per g Sepharose as compared to the amount of urate that was attached to the gel (Table 4.2.1.3.1), the capacity of Sepharose-xanthine was found to be only about 65% of the capacity of Sepharose-urate as the absorbent for uricase. No absorption was observed when uricase extract was passed through Sepharose-oxonate and Sepharose-cyanurate support. Even with very low flow rate no absorption of uricase was detected.

Uric acid has a very limited solubility in aqueous solution. At 4° only 3mM uric acid in 0.1M borate pH 9.0 remained in solution, and 5mM uric acid stayed in solution (in the same buffer) at 20°. The limited solubility resulted in precipitation forming in the protein containing eluate.

Xanthine could be dissolved in 0.1M borate pH 9.0 up to a concentration of 25mM. In experiments performed using 25mM xanthine solution, using the column elution technique, uricase was eluted in fairly large volume. Typically less than 40% adsorbed activity (70U) was recovered in the first 15 ml eluate.

25mM potassium oxonate in 0.1M borate pH 9.0 proved to be a more suitable eluant. (This concentration of oxonate was also limited by the limited solubility of the compound in aqueous solution.) Generally 60% absorbed uricase was eluted in the first 12 ml of eluant, and about 70% of the absorbed uricase was collected in 25 ml eluant.

Fig. 4.2.1.3.1 The capacity of Sepharose-ligand for bioaffinity binding of uricase. The procedure was as described in section 4.1.6.2a. Support with the highest ligand concentrations were used (Table 4.2.1.3.1). The percentage of total activity absorbed on Sepharose-urate (●) and Sepharose-xanthine (▲) and the percentage activity bound per ml of sample input onto Sepharose-urate (○) and Sepharose-xanthine (△) are illustrated.


% ACTIVITY BOUND PER ML.
100
80
60
40
20
0
TOTAL INPUT ACTIVITY (units)
0
20
40
60
80
100
120
140
160
180
% TOTAL ACTIVITY BOUND
100
80
60
40
20
0

As uricase-inhibitor eluates had to be separated by filtration through Sephadex G25, these values were actually calculated after the uricase had been eluted in the gel filtration procedure (section 4.1.7).

It was observed that recovery of enzyme was much higher (up to 90%) when small input of protein extract was applied to the affinity column (5-10 units of uricase).

Sepharose-urate support washed with eluant and 0.5M NaCl in buffer, could be reused for bioaffinity chromatography of uricase. There was an apparent decrease in the capacity of adsorption in the subsequent cycles (about 10% of the maximum capacity in the second cycle, and 30% in the third cycle).

Uricase solutions were concentrated by the ultra-filtration method described in section 4.1.8. A decrease in activity of around 30% was observed in the case of many fold increases in concentrations of proteins (10 fold). Uricase has been concentrated to 1 mg protein ml^{-1}.

Purified uricase was stored in 0.1M borate buffer pH 10.0 at 4°, without loss of activity in the period of up to 3 months.

Uricase stored in 0.1M borate pH 9.0 at 4°, apparently precipitated out after 2 or 3 days when the protein concentration was 0.1 $mg.ml^{-1}$ or greater.

Section 4.2.2 presents a summary of the final procedures adopted for the purification of uricase from procine liver.

4.2.2 Final procedure for the purification of uricase from porcine liver

1. Homogenisation of porcine liver

Fresh liver, or liver already thawed was cut into small pieces and weighed. 400 g liver was homogenised in 1ℓ of 0.1M borate, 1mM EDTA, and 1.5%(w/v) Triton x 100, pH 10, for 1 min. The suspension was stirred for 2h at 4°. 2 volumes of 0.1M borate, pH 10 containing 1mM EDTA were added to the homogenate and the suspension stirred for a further 30 min. The homogenate was then strained through muslin cloth. The residue was discarded.

2. Heat treatment

A 500 ml fraction of the diluted homogenate was placed in a 2ℓ conical flask and immersed in an 80° water bath. The flask was swirled and the temperature of the homogenate monitored. When the temperature of the homogenate reached 60°, the flask was immediately removed from the bath and stood at room temperature for 5 min. After 5 min the flask was immediately immersed in ice water and swirled to achieve rapid cooling, until the temperature of homogenate fell to less than 10°.

3. n-Butanol separation

n-Butanol was cooled at -20° overnight. n-Butanol and heat-treated homogenate (1:1,v/v) were mixed together. The alcohol

was gradually added to the homogenate with constant stirring.

The suspension was then centrifuged for 60 min, at 2000 x *g*. The butanol layer and the protein-lipid layer were discarded, while the aqueous layer was collected.

4. Ammonium Sulphate precipitation

Finely powdered ammonium sulphate crystals were gradually added to ice-cold protein extract (aqueous layer from butanol separation), until 40% saturation was achieved. After 1h standing, the suspension was centrifuged at 5000 x *g* for 30 min.

The precipitate which separated at the top layer was carefully recovered. Uricase was extracted from the precipitate using 0.1M borate 1mM EDTA pH 10. The precipitate was stirred in the buffer for 30 min and centrifuged at 5000 x *g* for 30 min. The supernatant contained uricase activity, and was recovered. The residue was re-extracted for any uricase remaining.

5. Bioaffinity chromatography

Affinity support was prepared as described in section 4.1.6.1. 2 g Sepharose-uric support was packed in a small cylindrical glass column (1 x 6 cm). The column was filled to a height of 5 cm, and the affinity gel was equilibrated with 0.1M borate, pH 9.0. The column was maintained at 4°.

Uricase extract was dialysed overnight in 0.1M borate pH 9.0. The dialysed extract was perfused through the gel bed at 0.4 $ml.min^{-1}$. Activity and protein content of the eluate was monitored. Sample input was terminated when the output (eluate) activity was about 10% of the sample input.

The gel bed was then washed with 0.1M borate buffer pH 9.0, containing 0.5M NaCl, to remove non-specifically adsorped proteins. The gel bed was then washed to remove the salt off the gel, using the equilibrating buffer.

Uricase was eluted from the affinity support by perfusing through the gel column 25mM oxonate (in 0.1M borate, pH 9.0) at 0.4 $ml.min^{-1}$. Fractions (4 ml) of eluant were collected. Uricase should be eluted in the initial fractions.

To determine the units of uricase recovered, the eluates were passed through Sephadex G25 (section 4.1.7).

Fig. 4.2.2.1 shows a typical adsorption and elution profine of uricase on a bioaffinity column. Table 4.2.2.1 summarises the purification procedures as described in this section.

Fig. 4.2.2.1 Adsorption and elution profile of uricase on the bioaffinity support. (O) denotes uricase activity, and (●) denotes protein concentration. Sample was applied at point A, buffer containing 0.5M NaCl at point B, equilibrating buffer at C, and eluant was applied at point D. 8 ml per fraction was collected. A total of 117 units of uricase was applied and 85 units were recovered in the eluates.

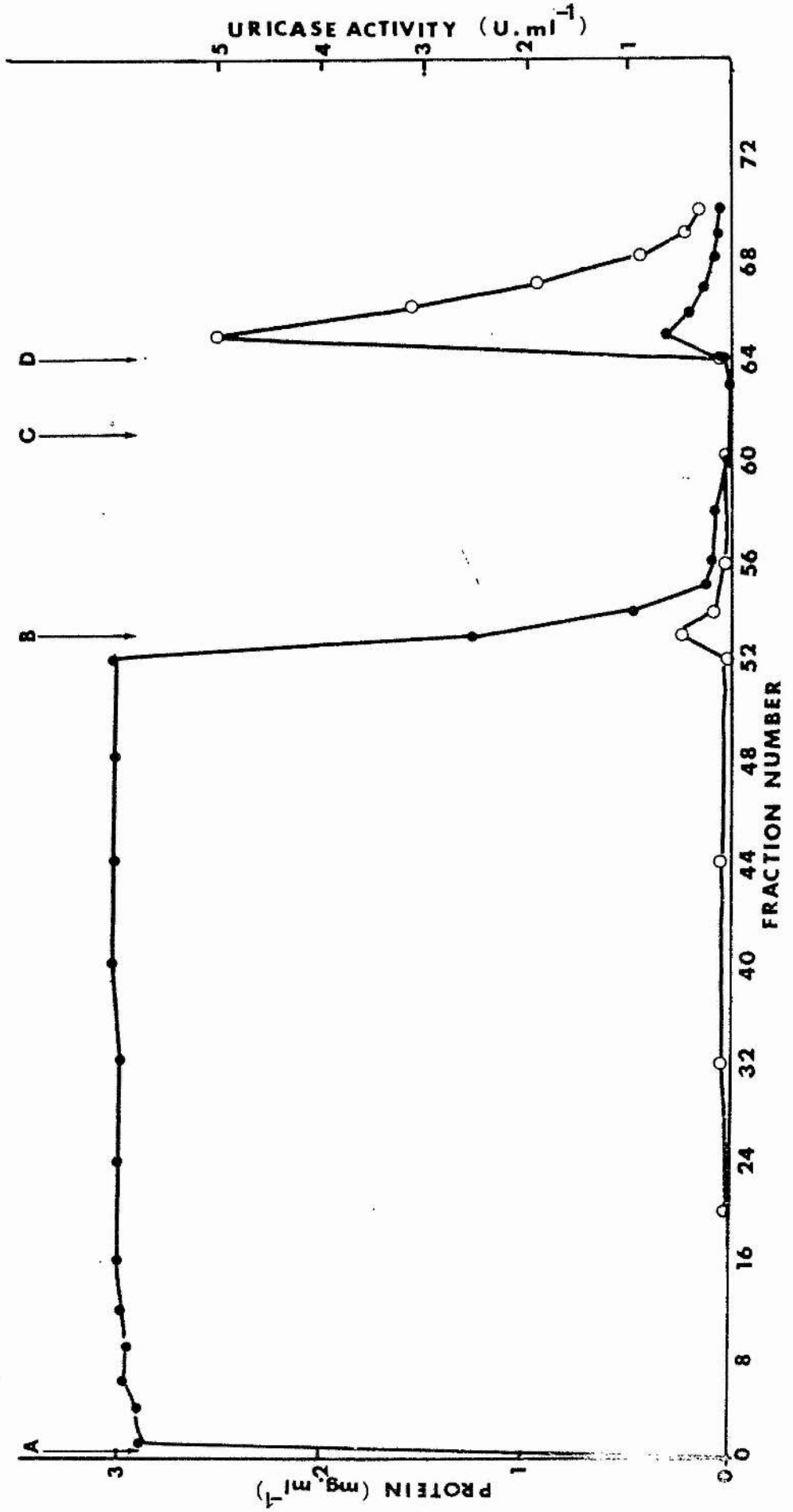

URICASE ACTIVITY (U.ml^{-1})
5
4
3
2
1
0
72
68
64
60
56
52
48
44
40
32
24
16
8
0
FRACTION NUMBER
D
C
B
A
3
2
1
0
PROTEIN (mg.ml^{-1})

Table 4.2.2.1 A summary of the extraction and purification of uricase from porcine liver. For further details of the procedure see section 4.2.2.

Procedure	Volume (ℓ)	Activity ($u.ml^{-1}$)	Activity Total(U)	Protein ($mg.ml^{-1}$)	Protein Total(g)	Sp.Act. ($U.mg^{-1}$)	Purification (fold)	% Activity recovered
Homogenation of liver (400 g), in 0.1M borate with 1.5% Triton X100, pH 10.	1.5	0.08	120	65	97.5	0.0012	1	
Dilution of homogenate	4.5	0.04	180	27	121.5	0.00148	1.2	
Heat treatment	4.3	0.075	322	20	86	0.00375	3.1	100
n-Butanol separation	3.7	0.078	287	8	29.6	0.00975	8.1	89
40% Ammonium sulphate precipitation.								
First extract	0.6	0.4	240					
Second extract	0.195	0.095	18.5					
Total extract (combined)			258.5	3.1	2.5	0.105	87.5	80
Bioaffinity chromatography								
Sepharose-urate	0.221	0.82	181	0.1	0.0221	8.2	6833	56

4.2.3 Characterisation of purified uricase

4.2.3.1 Gel filtration on Sephadex G200

Gel filtration on Sephadex G200 was carried out as described in section 4.1.8. Void volume of the gel bed was found to be 40 ml. 2 ml uricase (0.5 $mg.ml^{-1}$) was applied to the gel column, and uricase was eluted in about 60 ml elution volume. About 10% of input activity was lost in the filtration procedure. No increase in specific activity was found.

4.2.3.2 Ultra-violet spectrum of purified uricase

Uricase solution (0.2 $mg.ml^{-1}$) was dialysed into 0.1M borate buffer pH 10 for 16h. This solution was scanned on the Beckman DB GT spectrophotometer in the range of 320-220nm wavelength. The spectrum was recorded on the PYE UNICAM AR45 recorder, as shown in Fig. 4.2.3.2.1. The maximum absorption of purified uricase is found to be at 277nm.

4.2.3.3 Polyacrylamide disc-gel electrophoresis

Two different methods were adopted in studying the characteristics of purified uricase in polyacrylamide electrophoresis.

Fig. 4.2.3.3.1 shows a photograph of electrophoresis gels after protein and activity staining obtained by the method described in section 4.1.10.1. There was essentially a single protein band which corresponded to the band of activity stain. No

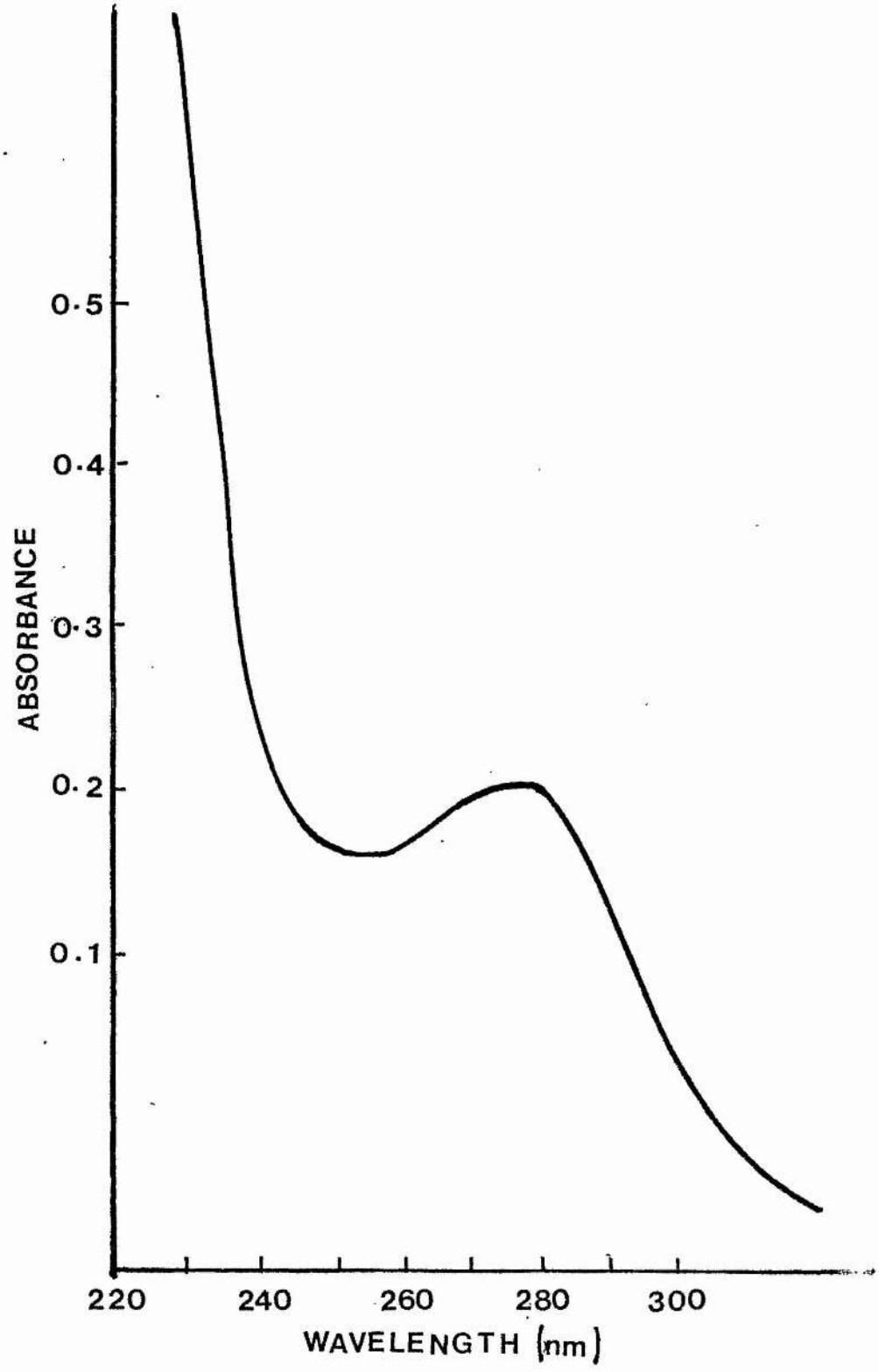


Fig. 4.2.3.2.1 Ultra-violet spectrum of purified uricase.

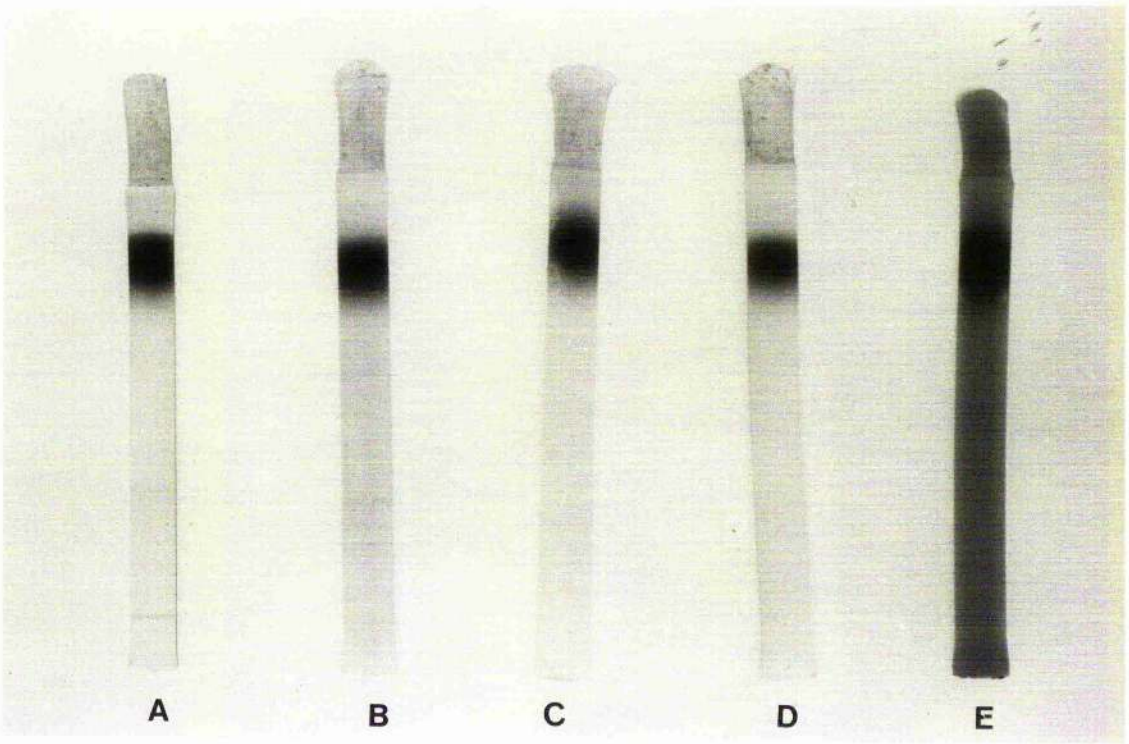


Fig. 4.2.3.3.1 Disc-gel electrophoresis patterns of purified uricase (section 4.1.10.1).

A: 20µg uricase, electrophoresis ran at pH 8.5.

B: 10µg uricase, electrophoresis ran at pH 8.5

C: 10µg uricase (after Sephadex G200 filtration), electrophoresis ran at pH 8.5.

D: 10µg uricase, electrophoresis ran at pH 9.0.

E: 20µg uricase, electrophoresis ran at pH 8.5; activity stain.

significant difference was observed when the electrophoresis was carried out at pH 8.5 and 9.0. Enzyme samples obtained after Sephadex G200 filtration showed identical results with uricase samples obtained from Sephadex G25.

Gel electrophoresis in the presence of SDS also resulted in a single protein band migrating down the gel. However no uricase activity was detected on the gel by activity staining (section 4.1.11).

It was observed that this single protein band could be detected even without pre-treatment of the uricase sample with SDS-glycine buffer and 2-mercaptoethanol (section 4.1.10.2).

4.2.3.4 Molecular weight estimation of purified uricase

Molecular weight was initially estimated by the SDS-polyacrylamide disc-gel electrophoresis (section 4.1.10.2). By applying standard proteins to the electrophoresis, the migration of different proteins down the gels could be compared (Fig. 4.2.3.4.1). A plot of molecular weights against the corresponding mobility of the protein bands is shown in Fig. 4.2.3.4.2. The molecular weight of purified uricase was estimated using this plot.

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance moved by tracking dye}}$$

Molecular weight of uricase estimated by this method was in the range 32-34 x 10^3.

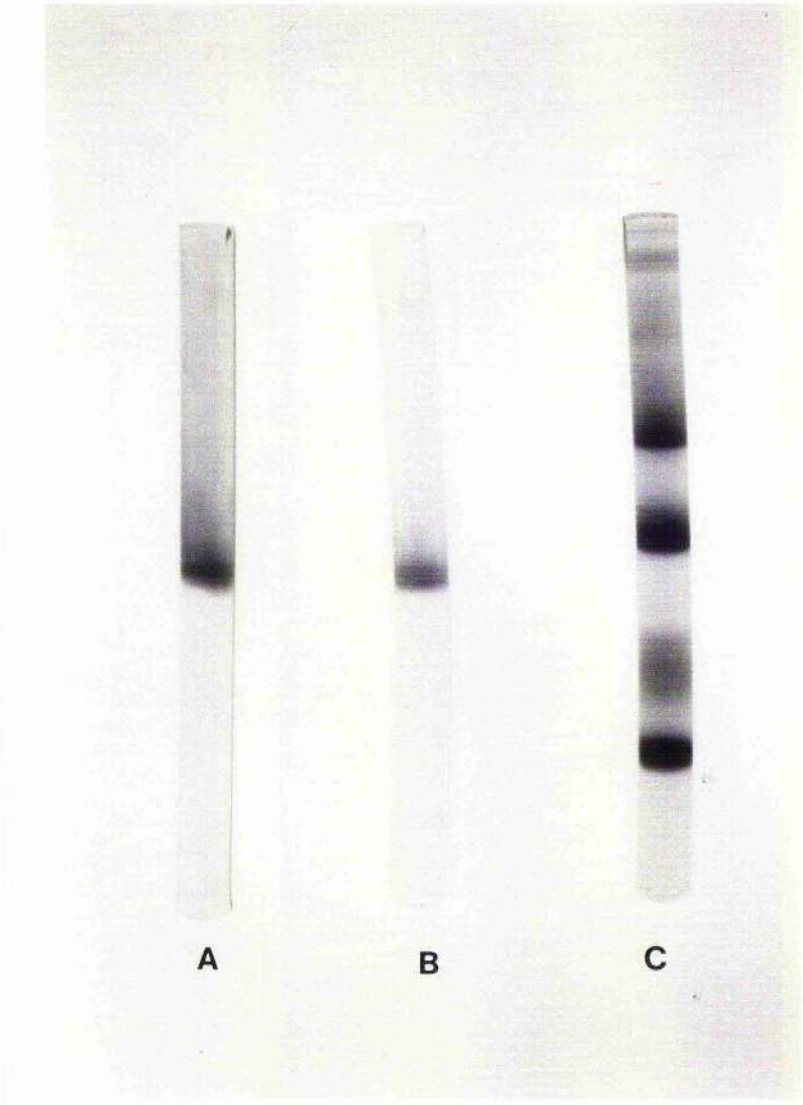


Fig. 4.2.3.4.1 SDS-acrylamide disc-gel electrophoresis patterns. 10µg uricase samples, untreated (A) and treated (B) were applied to the gels. (C) denotes the gel with standard proteins. For further details see section 4.1.10.2.

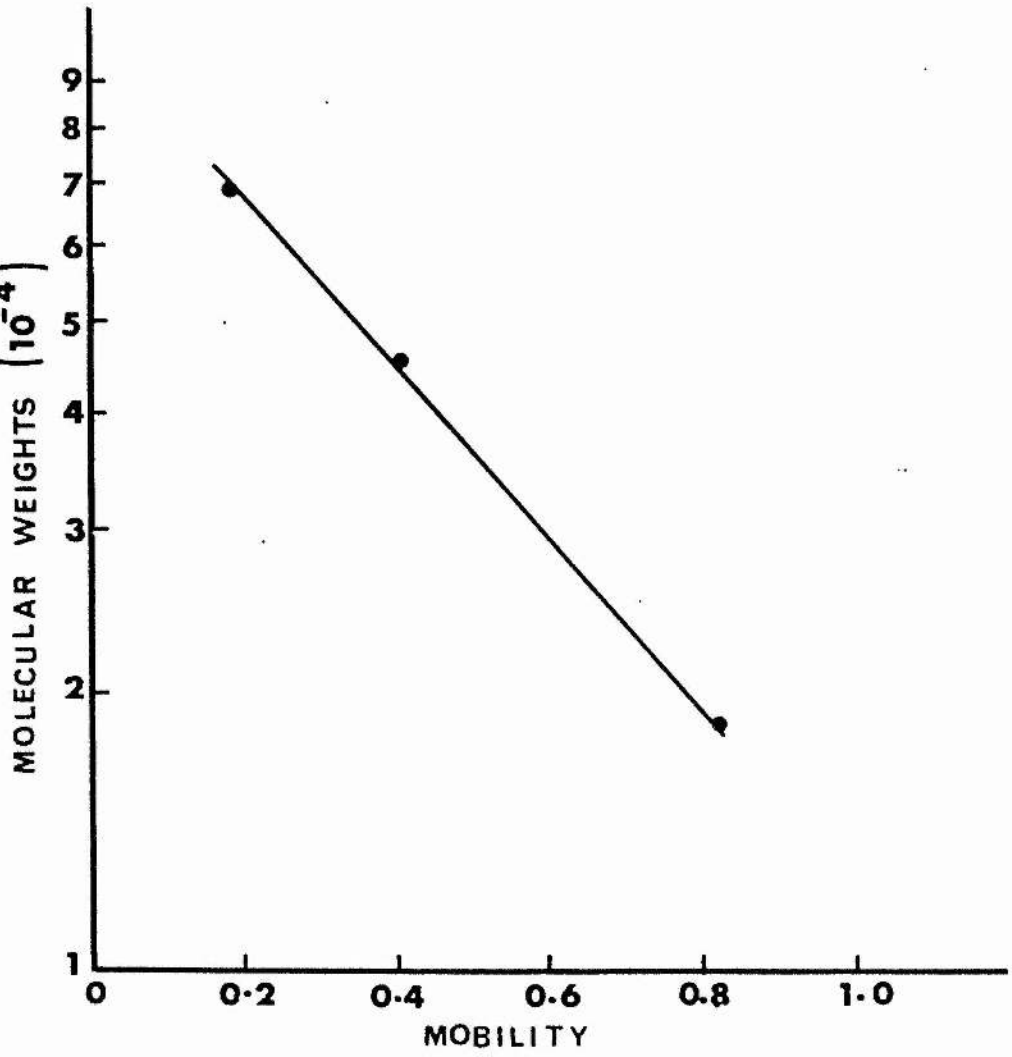


Fig. 4.2.3.4.2 Calibration plot for the estimation of molecular weight by SDS-acrylamide disc-gel electrophoresis.

Molecular weight was also estimated by the ultra-centrifugation method described in section 4.1.12. Fig. 4.2.3.4.3 shows the optical pattern obtained from the ultracentrifugation method. Measurement of the interference pattern and computer analysis of the data into a programme, the molecular weight of the protein sample could be estimated.

Fig. 4.2.3.4.4 shows a plot of inverse sigma $(\frac{1}{\sigma})$ against concentration (proportional to the fringe displacement), where,

$$\sigma = \frac{M_i(1 - \bar{v}\rho)\omega^2}{RT}$$

is the "reduced molecular weight", M_i is the molecular weight for the protein, $\bar{v}_i$ is its partial specific volume, ρ is the solution density, ω is the angular speed, R is the gas constant and T is the absolute temperature.

From the computer result,

$$\frac{RT}{(1 - \bar{v}_i\rho)\omega^2} = 36903$$

The values of sigma along the concentration gradient was also computed. From the plot (Fig. 4.2.3.4.4) the value of sigma at zero concentration was postulated, and M_i was calculated as 1.34×10^5.

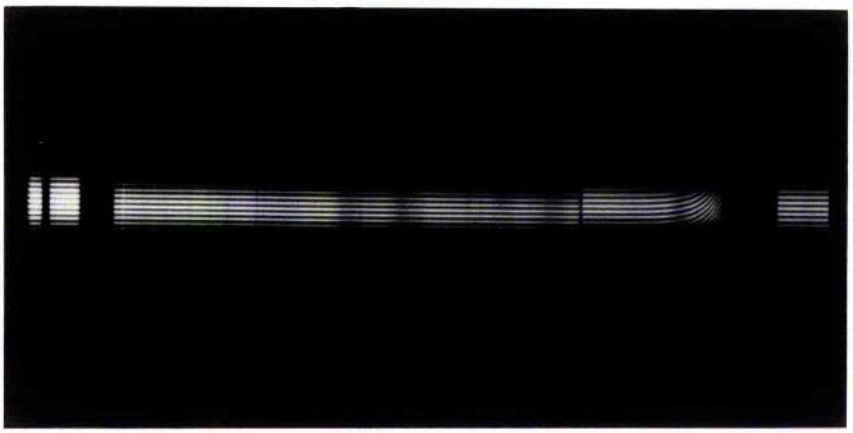
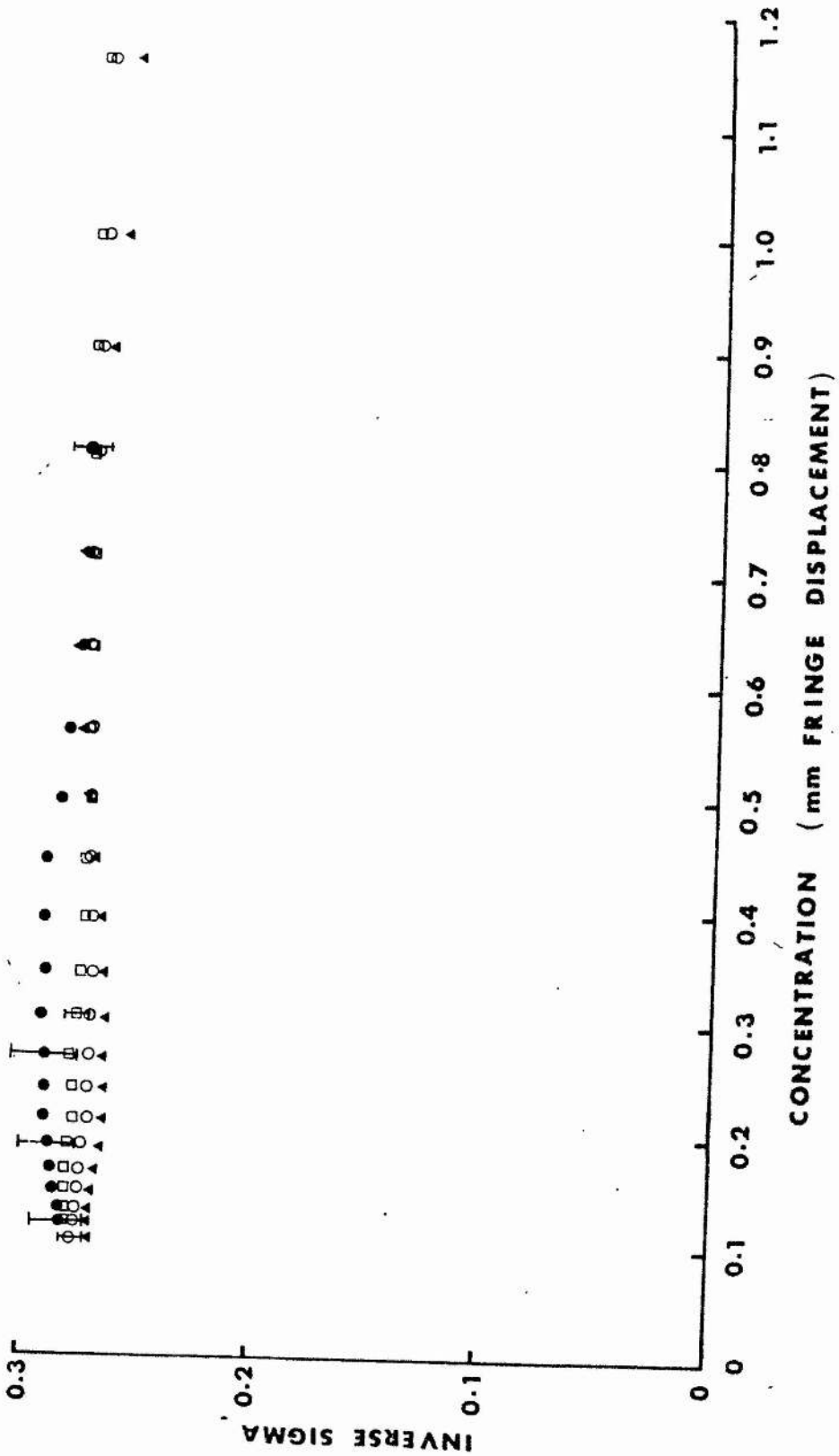


Fig. 4.2.3.4.3 Interference pattern obtained by the ultra-centrifugation method.

Fig. 4.2.3.4.4 Plot of inverse sigma against concentration, for the estimation of molecular weight.


INVERSE SIGMA
0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
CONCENTRATION (mm FRINGE DISPLACEMENT)

4.2.4 Determination of the kinetic parameters of purified uricase

4.2.4.1 Determination of K_m and V_{max}

The K_m and V_{max} of purified uricase were determined from initial velocity data by the graphical method of Lineweaver and Burk (121) and also by fitting initial velocity data to the hyperbolic Michaelis-Menten equation using an ALGOL-W computer programme (122).

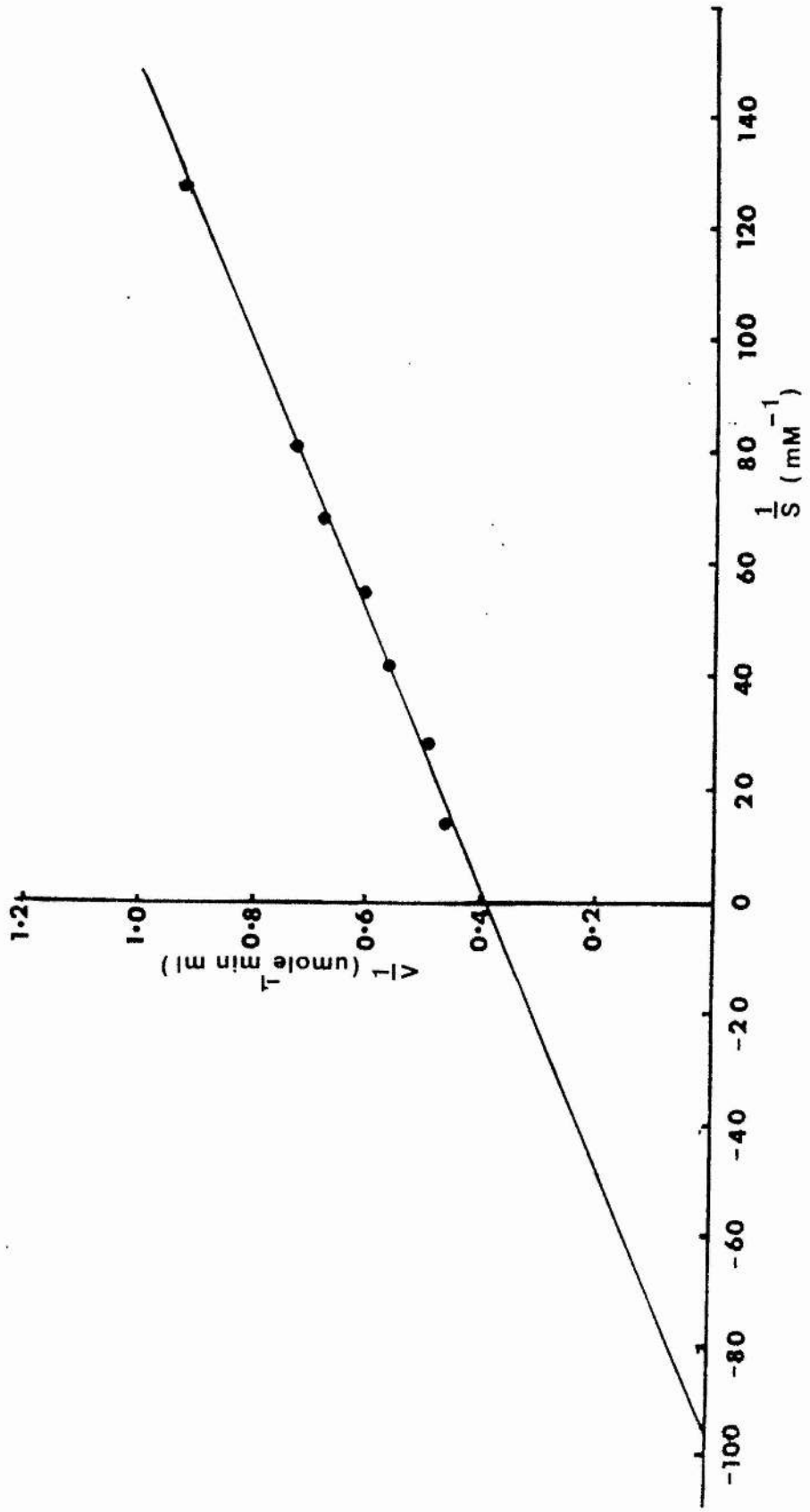
The initial velocity for a range of substrate concentration of 0.004 - 0.08mM were measured by the oxygen electrode method (section 3.1.1). The reactions were carried out in 0.1M borate buffer pH 8.6 at 25°.

The double reciprocal plot for the determination of K_m and V_{max} is shown in Fig. 4.2.4.1.1. Table 4.2.4.1.1 shows the results obtained by the two methods.

Methods	V_{max} ($U.mg^{-1}$)	K_{max} (M)
Double-reciprocal plot	12.6	1.04×10^{-5}
ALGOL-W computer analysis	12.56 ± 0.28	$1.03 \pm 0.06 \times 10^{-5}$

Table 4.2.4.1.1 K_m and V_{max} values for purified uricase.

Fig. 4.2.4.1.1 Double-reciprocal plot for the determination of K_m and V_{max}. Each velocity value was the mean of three determinations. The best fit line was drawn through the points.


1·2
1·0
0·8
0·6
0·4
0·2
$\frac{1}{V}$ (umole^{-1} min ml)
-100
-80
-60
-40
-20
0
20
40
60
80
100
120
140
$\frac{1}{S}$ (mM^{-1})

4.2.4.2 pH profile of purified uricase

The pH-activity profile of uricase was determined by assaying the enzyme in a range of suitable buffers. The rate of reaction was monitored by the method described in section 3.1.1.

A pH range of 6.5-11.0 was used with different buffer systems. The results were plotted with activity expressed as the percentage of maximum activity against pH as shown in Fig. 4.2.4.2.1. The optimum pH was to be in the range of 8.9 to 9.1.

4.2.4.3 Thermal denaturation of uricase

0.5 ml aliquots of uricase (0.1 and 0.2 $mg.ml^{-1}$) in 0.1M borate buffer pH 10.0 were incubated in a constant temperature bath in the range of 25°-60°, for set time periods (within the range 5-60 min). At the end of the incubation period the enzyme was cooled in an ice bath and 0.1 ml aliquots were assayed for residual activity.

A plot of residual activity (expressed as the percentage of maximum activity) against incubation period is presented in Fig. 4.2.4.3.1.

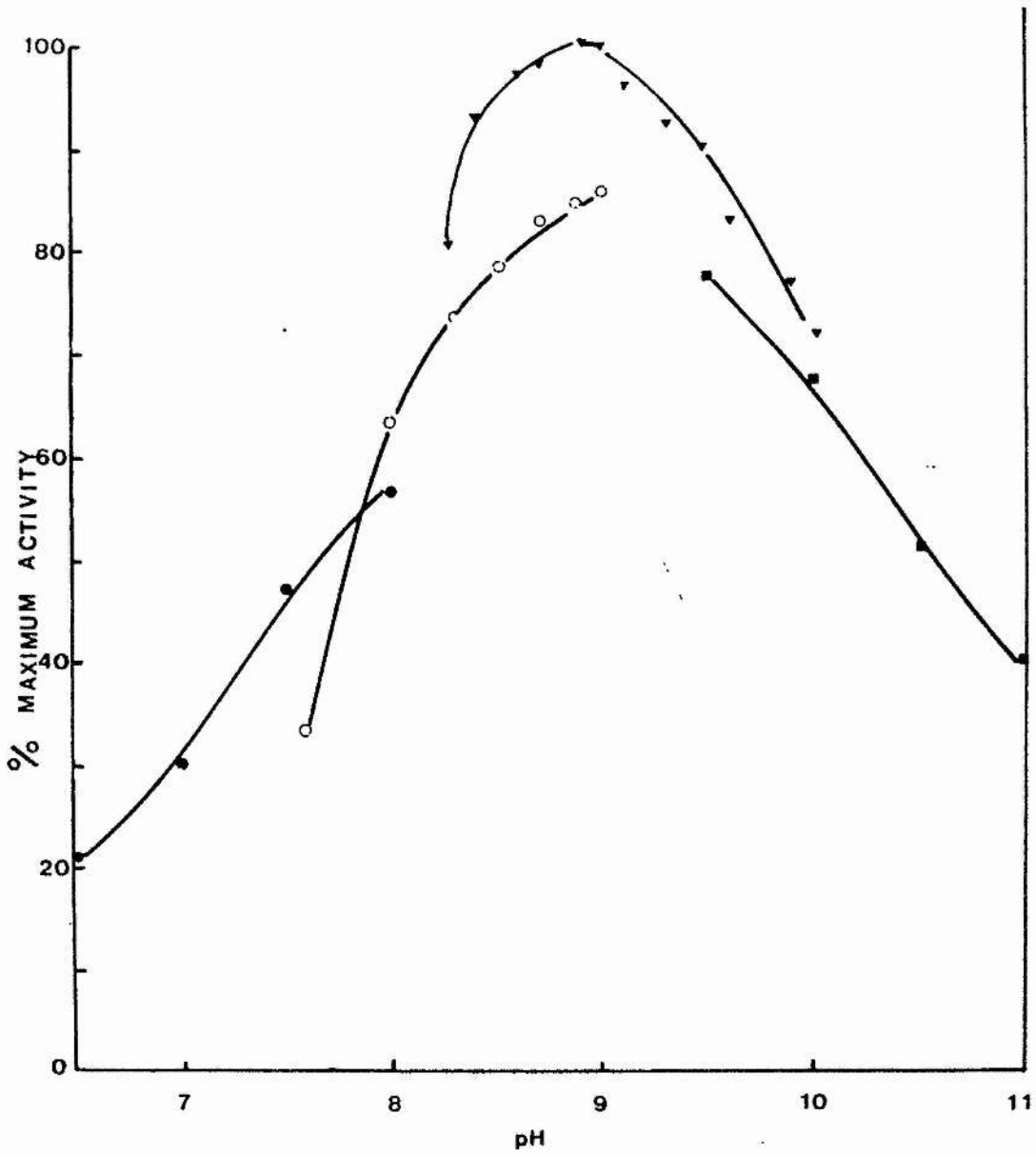


Fig. 4.2.4.2.1 pH profile of purified uricase. The enzyme was assayed in phosphate (●), TRIS (O), borate (▼) and glycine buffers (◆).

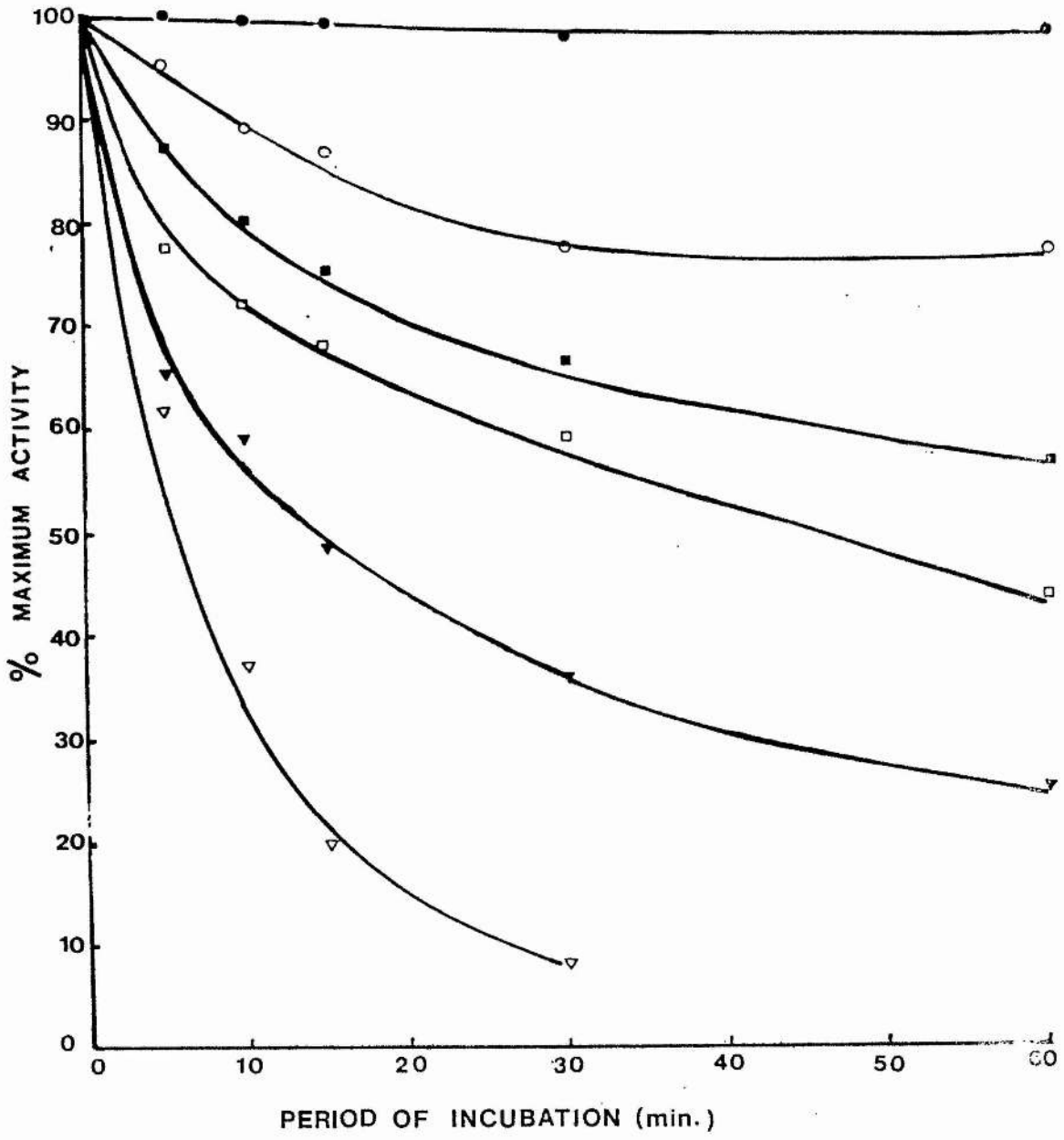


Fig. 4.2.4.3.1 Thermal denaturation of uricase. The enzyme was incubated at 25° (●), 37° (○), 45° (■), 50° (□), 55° (▼) and 60° (▽).

4.3 Discussion

The initial stages of the purification process were devised for obtaining protein extract with high uricase content, suitable for application to the bioaffinity procedure. With consideration that the process should be adaptable to large scale techniques, the procedures were developed with the following objectives in mind.

1. To maximise the extraction of the enzyme from liver.
2. To minimise the volumes to be handled during the purification process.
3. To eliminate high speed centrifugation procedure in the process.

Alkaline pH solutions were chosen for extracting uricase due to its stability and solubility in such pH ranges (111,112). The difference in the total units of extracted uricase in solution of pH 10 and 11 was rather minimal, although Yokota (43) obtained more significant differences. The presence of Triton X100 and n-butanol increased the solubility of proteins, but the primary role of the detergent and alcohol may have been in the disintegration and dissociation of the uricase from the nucleod cores of the microbodies.

The presence of n-butanol in the extracting buffer may not be suitable especially if the homogenate were to be exposed to heat. Leone (44) and London and Hudson (45) did however utilise warm n-butanol-buffer solution for extracting uricase

but the recoveries were rather low.

Triton X100, a neutral detergent, was found to be more suitable for uricase extraction in preference to cationic detergents(110) although Otta and Bertini (111), using Hyamine 2389, a cationic detergent, found the contrary. The presence of Triton X100 increased the extraction of uricase and protein solubilisation. In fact, varying concentration of Triton X100 affected the solubility of proteins, and excessive amount of the detergent only served to increase protein content without increasing uricase content. A suitable concentration of the detergent in the extraction procedure should be used to obtain uricase with high specific activity. Truscoe (110) used 1.5% Triton X100 in the extracting buffer to obtain high specific activity enzyme, but in this work 0.5% detergent in buffer was used with good effect. Obviously the volume of extracting buffer was the limiting factor.

Heat treatment of the homogenate may further promote the dissociation of uricase from the crystalloid cores, though the increase in uricase content may also be attributed to the destruction of inhibitory agents.

The quantity of uricase extracted (over 300 units from 400 g liver) is high compared to previous preparations (41-43).

The n-butanol separation technique provided a mean of separating most of the unwanted proteins and lipids. Significantly this was the first step in which centrifugation was utilised. Sufficient separation of the phases was obtained under low speed

centrifugation, and the uricase active layer could be collected with little loss in activity.

Ammonium sulphate precipitation is a commonly used procedure for purification and concentration of enzyme solutions. Mahler et al. (42) used a 2-step precipitation procedure to remove non-uricase protein in the first instance and to precipitate uricase in the second. A similar principle was followed here but with a single precipitation step followed by re-extraction of the precipitate for uricase. This procedure was more convenient as there is a smaller volume in the re-extraction step.

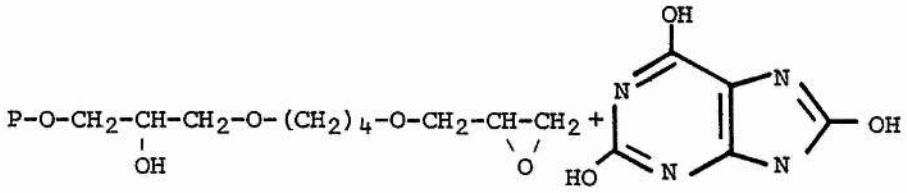
The behaviour of uricase on DEAE-cellulose may be explained by the high affinity between opposite charges of the enzyme and the ion exchangers, indicating that uricase molecules are polyanionic under the conditions studied. Truscoe (110) showed that uricase was irreversibly inhibited by cationic detergents, comprising quarternary ammonium salts, due to the formation of complexes. In another work, long chain alkyl groups seemed to be involved in the inhibitory effect (123). Nevertheless the formation of these complexes may contribute to the inability to elute uricase from the ion exchanger. DEAE-cellulose chromatography achieved some purification of uricase, but the recovery was too low for this technique to be of much use.

The degree of purification and high recovery achieved by the bioaffinity chromatography process explain the increasing

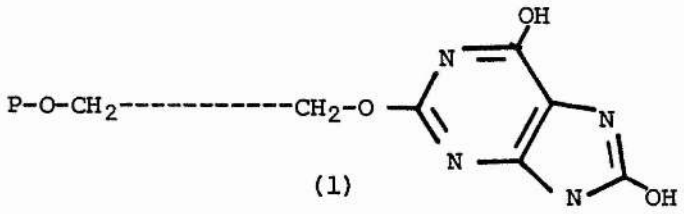
popularity of this technique in recent years in enzyme purification.

Uric acid, the substrate and xanthine, a relatively strong purine inhibitor (K_i - 1.2 x 10^{-5}M) of uricase (55) were used as ligands. Prodovich (124) showed that s-triazines, oxonic acid (K_i - 1 x 10^{-5}M) and cyanuric acid (K_i - 3 x 10^{-5}M) were also competitive inhibitors of uricase, and because of their greater solubility in aqueous solutions were also considered as ligands. All these compounds have chemical groups which may be suitable for coupling to oxirane activated supports. Fig. 4.3.1 shows the possible configurations of these ligands and supports after coupling.

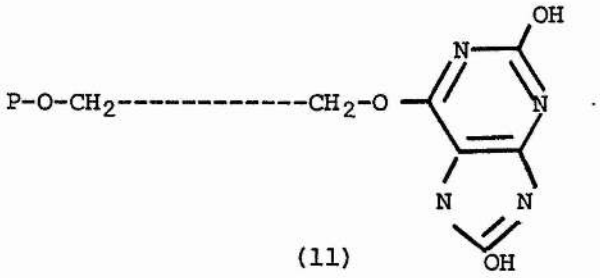
However, the coupled s-triazines were found to be unsuitable ligands. Uricase shows no affinity for these supports under the conditions investigated. It was thought that the inhibitory effect was promoted by the s-triazine ring, which was similar to part of the purine ring, and substituting one of its available hydroxyl groups would not significantly alter its inhibitory property. Furthermore, substituted pyrimidines have also been shown to possess inhibitory effects on uricase (125). It seems that the presence of long chain groups substituted into the s-triazine ring is detrimental to the inhibitory property of these compounds. Loss of inhibitory capacity may be due to inaccessibility of the s-triazine molecule to the active site of the enzyme. Curling up of the long chain spacer 'arm', thus masking the ligands cannot be ruled out.



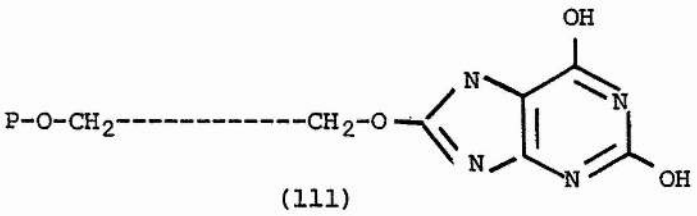
uric acid



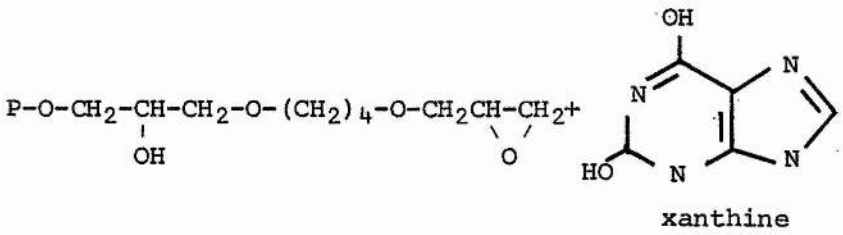
(I)



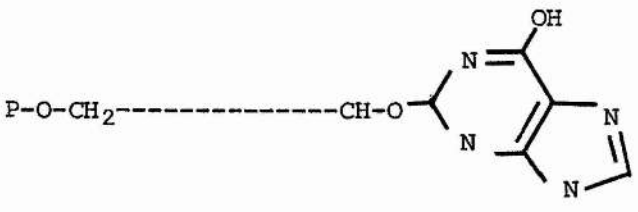
(II)



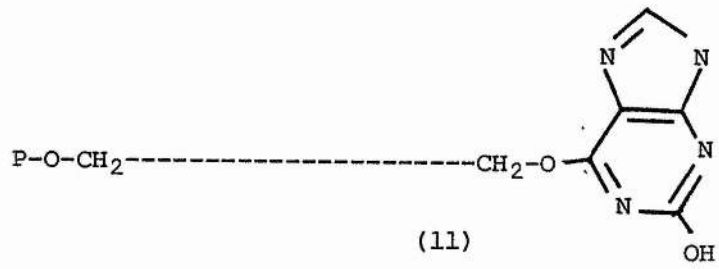
(III)



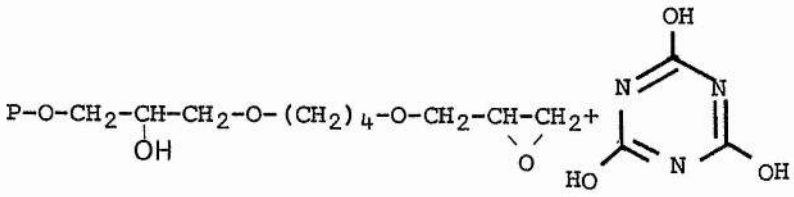
xanthine



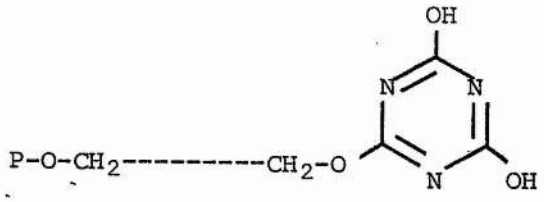
(I)

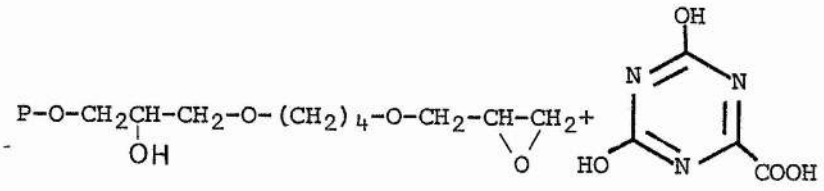


(11)



cyanuric acid





Oxonic acid

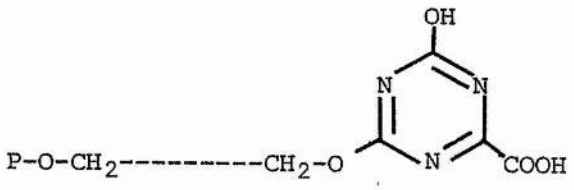


Fig. 4.3.1 Possible configurations for Sepharose-ligand. coupling.

Successful adsorption was achieved with coupled urate and xanthine. It was notable that more xanthine was coupled per g support but the adsorption capacity was less than the urate-support complex. Obviously the substituted urate has greater affinity for uricase than the substituted xanthine, although the chemistry of coupling may be more suitable to the xanthine molecule.

High enzyme recovery can be achieved by the bioaffinity technique. In this work, the final recovery (ca.70%) may be lower than expected but the enzyme was not measured until the inhibitor was removed from the eluate by gel filtration. Exposure of uricase to the Sephadex surfaces may have denatured some of the uricase (47,110). Filtration through Sephadex G200 resulted in some loss of activity. The long exposure of uricase to the surfaces of the Sepharose support during the bioaffinity chromatography step cannot be disregarded as a factor in the denaturation of the enzyme.

Inhibitors may not be ideal for elution of enzyme from affinity supports. Complete separation of the enzyme from the inhibitor may be difficult due to the affinity of the inhibitor for the enzyme molecule. Apparent low enzyme activity may be measured unless the inhibitors can be completely dissociated from the enzyme molecules. Substrate(s) or co-substrate(s) may be better eluting agents, but in the case of uricase, the use of its substrate was limited by its low solubility.

High purity enzyme was obtained by this purification process. The average specific activity of 8 $U.mg^{-1}$ is high compared to most commercial preparations, and the recovery of enzyme is significantly higher than those obtained by many previous workers (especially when the total units of uricase available is also higher).

Homogeneity of the purified uricase was shown by gel electrophoresis where a single protein band was obtained. Interestingly, a single protein band was also obtained with lower specific activity uricase (43) possibly pointing to the isolation of partially denatured uricase.

The clear interference pattern of the Rayleigh optics in the ultracentrifugation system provided further evidence of the homogeneity of the enzyme preparation.

The ultra-violet spectrum of the purified uricase is similar to those obtained previously in earlier work (43,57).

The V_{max} of the purified uricase is found to be 12.6 $U.mg^{-1}$, and the K_m value is about $1 \times 10^{-5}M$. Yokota (43) obtained V_{max} and K_m values of 5.4 $U.mg^{-1}$ and $5.8 \times 10^{-5}M$ respectively. K_m values closer to the value obtained here were obtained by Baum *et al.* (56) ($1.7 \times 10^{-5}M$), Fridovich (124) ($0.5 \times 10^{-5}M$) and Sedor and Sander (125) ($0.78 \times 10^{-5}M$). Obviously the variations may be attributed to the non-standardised assay conditions, such as

buffer, temperature and substrate range. Variations in values can also be affected by the oxygen concentration in the assay solution.

The pH optimum of the purified uricase is about 9, a value corresponding to that obtained by Baum et al.(56) and is also close to the value obtained for bovine kidney uricase (44). However, Yokota (43) obtained a pH optimum of 9.6 for porcine liver uricase.

Two different values of molecular weights obtained can be attributed to the dissociation of the uricase molecule. The 'true' molecular weight of the native uricase is about 134,000, a value comparable to those obtained by Mahler (57), Leone (44) and Pitts et al.(126).

Pitts et al.(126) showed that uricase comprised four equal sub-units of molecular sub-units of molecular weight 32,000, a value obtained in this work, obtained by the SDS-gel electrophoresis. The absence of activity on the protein band on the gel indicates the loss of activity after dissociation. It was observed that dissociation could be achieved during electrophoresis without prior treatment, whereas Pitts et al. found that it required prolonged heating in SDS for dissociation to occur.

Pitts et al.(126) activated uricase in glycine buffer showed that uricase was fairly stable up to 47°. In this work, uricase appeared to be less stable in borate buffer, the enzyme being stable up to 25°. Apparently, the enzyme is much more stable in glycine buffer.

5. IMMOBILISATION OF URICASE ONTO NYLON TUBE

5.1 Methods

5.1.1 Activation of nylon tube by O-alkylation

5.1.1.1 Preparation of triethyloxomium tetrafluoroborate (TOTFB)

The method used is based on that described by Meerwein (127). The reaction is illustrated in Fig. 5.1.1.1.1.

Procedure

Boron trifluoride diethyletherate (25 g) was added to 200 ml dry diethyl ether in a round bottomed flask fitted with a reflux condenser. The solution was brought to reflux and 12.5 g 1-chloro-2:3-epoxypropane in 50 ml diethyl ether was gradually added to the solution and the mixture stirred under reflux for 2h. TOTFB precipitated as white crystals and was washed thoroughly with (4 x 100 ml) diethyl ether. The precipitate was dissolved in 200 ml dry dichloromethane and used within 24h of preparation.

5.1.1.2 O-alkylation of nylon tube

This procedure was optimised by Noy (92).

Procedure

Nylon tubings (2m lengths) filled with TOTFB solution (section 5.1.1.1) and sealed end to end were incubated at 25° for 15 min. The alkylation was terminated by washing with dry dichloromethane.

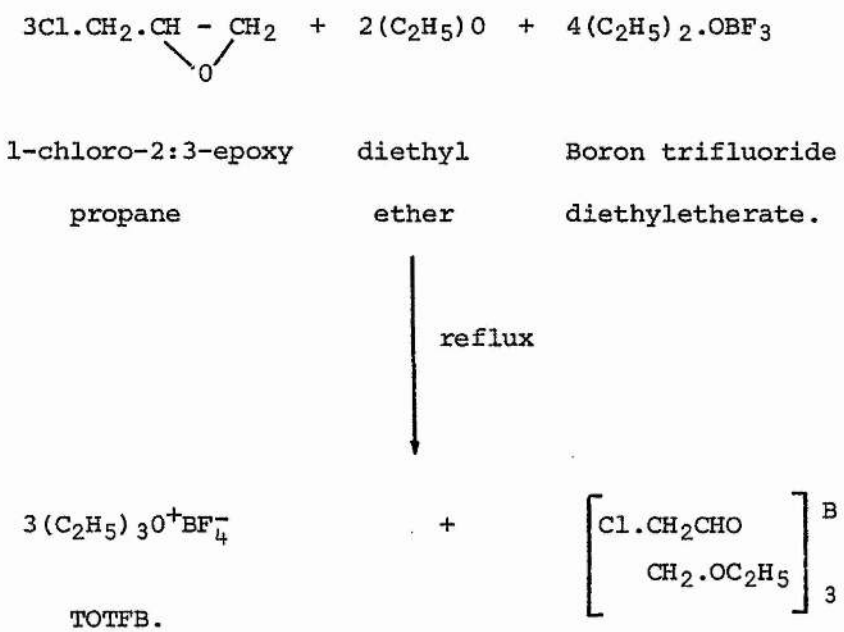


Fig. 5.1.1.1.1 Preparation of TOTFB as described in section 5.1.1.1.

5.1.1.3 Attachment of spacer to O-alkylated nylon tube

Reagents

1. Diaminoethane, 2M in methanol.
2. Adipid hydrazide, 4%(w/v) in formamide.
3. PEl, 20%(v/v) in methanol.

Procedure

O-alkylated nylon tube as prepared in section 5.1.1.2 was filled with appropriate spacer solution, sealed end to end and was incubated at room temperature. Excess amine or hydrazide was removed by washing with a large volume of water.

5.1.1.4 Coupling of enzyme to amine- or hydrazide substituted nylon tube

a. Reactivation of amine or hydrazide substituted nylon tube

i. Glutaraldehyde coupling

Procedure

Amine or hydrazide substituted nylon tube was perfused with 5%(w/v) glutaraldehyde in 0.2M borate buffer pH 8.5. The solution was recycled through the nylon tube for 15 min. The tube was free of excess glutaraldehyde by perfusion with 0.5M NaCl in 0.2M borate buffer pH 8.5. The tube was then washed through with the coupling buffer and used for enzyme coupling immediately.

ii. Bisimidate coupling

Amine or hydrazide substituted nylon tube was perfused with dry methanol and sucked dry by suction pump. The tube was recycled with a solution comprising 4%(w/v) dimethyl suberimidate, in 40%(v/v) N-ethylmorpholine in methanol. After 10 min the tube was washed dry with methanol and then used for enzyme coupling immediately.

b. Enzyme coupling to reactivated nylon tube

Nylon tube reactivated by glutaraldehyde or bisimidate (section 5.1.1.4a) was filled with enzyme solution to be immobilised.

Typically glucose oxidase (1 $mg.ml^{-1}$) in 0.2M borate buffer pH 8.5 was incubated in an activated nylon tube at 4° for 3h. The activity of pre- and post-coupling enzyme solution was assayed as described in section 3.1.2. The protein concentration of the solutions was assayed according to the methods described in section 3.2 or monitored at 280nm.

Unbound enzyme was washed from the nylon tube with buffer containing 0.5M NaCl.

5.1.2 Glutaraldehyde activation of nylon

5.1.2.1 Optimisation of conditions of activation for maximum activity retention

Glutaraldehyde has been used as a coupler to attach enzymes to amino-derivatised nylon tube. Optimum conditions for this reaction was studied by Onyezili (128). It was found that under certain conditions glutaraldehyde was able to attach directly to the nylon polymer, to produce reactive centres. These conditions were investigated.

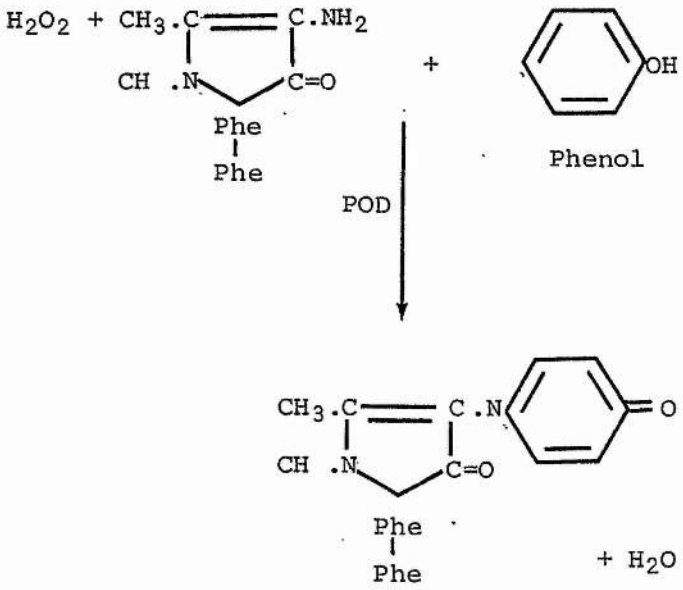
Glucose oxidase (GOD) was initially used as an index to assess the effect of varying conditions of activation. This enzyme was chosen, as it had been immobilised to nylon tube successfully, and it was available at fairly low cost.

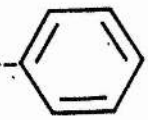
The activity of GOD on the nylon tube was assessed by incorporating the immobilised enzyme tube into a standard Technicon AA1 continuous flow analyser (AutoAnalyser) where it was perfused with substrate at a fixed flow rate and the amount of product formed was measured. At low percentage conversion the product would be proportional to the enzymic activity.

The activity of GOD was determined by the method described by Trinder (129). GOD catalysed the oxidation of glucose to yield gluconic acid and hydrogen peroxide. Hydrogen peroxide was then allowed to oxidise 4-aminophenazone (APZ) and phenol in a reaction catalysed by peroxidase (POD). A reddish pink

quinone is formed, this product being proportional to the glucose oxidised.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{GOD} \beta\text{-D-gluconic acid} + H_2O_2$$



Note: Phe 

Protein coupled was monitored by the absorption at 280nm of the pre- and post-coupling solutions.

Reagents

1. 0.1M phosphate pH 7.0 containing 0.05%(v/v) Triton X100 and 0.33M Na_2SO_4.

2. Colour reagents.

Reagent A: 1 g phenol was dissolved in 0.1M phosphate and made up to 1ℓ.

Reagent B: 60 mg APZ and 2 mg POD were dissolved in 0.1M phosphate pH 7.0 and the solution made up to 100 ml.

3. Glucose standard.

25mM glucose solution was made in saturated benzoic acid.

Procedure

Nylon tube (1m length) was coiled around a perspex cylinder and filled with glutaraldehyde solution. The tube was then sealed end to end and immersed in a constant temperature bath for a set period of time. The tube was washed clear of excess glutaraldehyde with 0.5M NaCl solution and subsequently filled with PEI solution and incubated at room temperature for 2h. The PEI-derivatised nylon tube was washed free of excess unbound PEI with a large volume of water.

Glutaraldehyde coupling of enzyme (section 5.1.1.4a, i) was utilised for immobilising GOD to the PEI-derivatised nylon tube.

GOD (1 $mg.ml^{-1}$) in 0.2M borate buffer pH 8.5 was incubated in the reactivated tube at 4° for 3h.

The tube was thoroughly washed with 0.5M NaCl in 0.1M phosphate pH 7.0, to remove non-covalently bound protein.

The immobilised enzyme tube (25 cm) was incorporated into the flow system as shown in Fig.5.1.2.1.1.

Conditions for glutaraldehyde activation of nylon were varied to obtain the optimal conditions for enzyme immobilisation.

a. Variation in glutaraldehyde concentration

Glutaraldehyde solution (25%, w/v) was diluted to varying concentration ranging from 1.25 to 18.5% in 0.5M borate pH 9.0. Nylon tube filled with the glutaraldehyde solution was incubated for 10 min in a 90° bath.

b. Variation in the period of incubation

Nylon tube was filled with 18.5%(w/v) glutaraldehyde in 0.5M borate buffer pH 9.0 and incubated at 90° for 2.5-30 min.

c. Variation in temperature of incubation

Nylon tube was filled with 18.5%(w/v) glutaraldehyde in 0.5M borate buffer pH 9.0 and immersed in constant temperature baths at temperatures ranging from 0-90° for 15 min.

d. Variation of pH of glutaraldehyde solution

Nylon tube was filled with 18.5%(w/v) glutaraldehyde in 0.5M buffer of different pH values. The tube was incubated for 15 min at 90°.

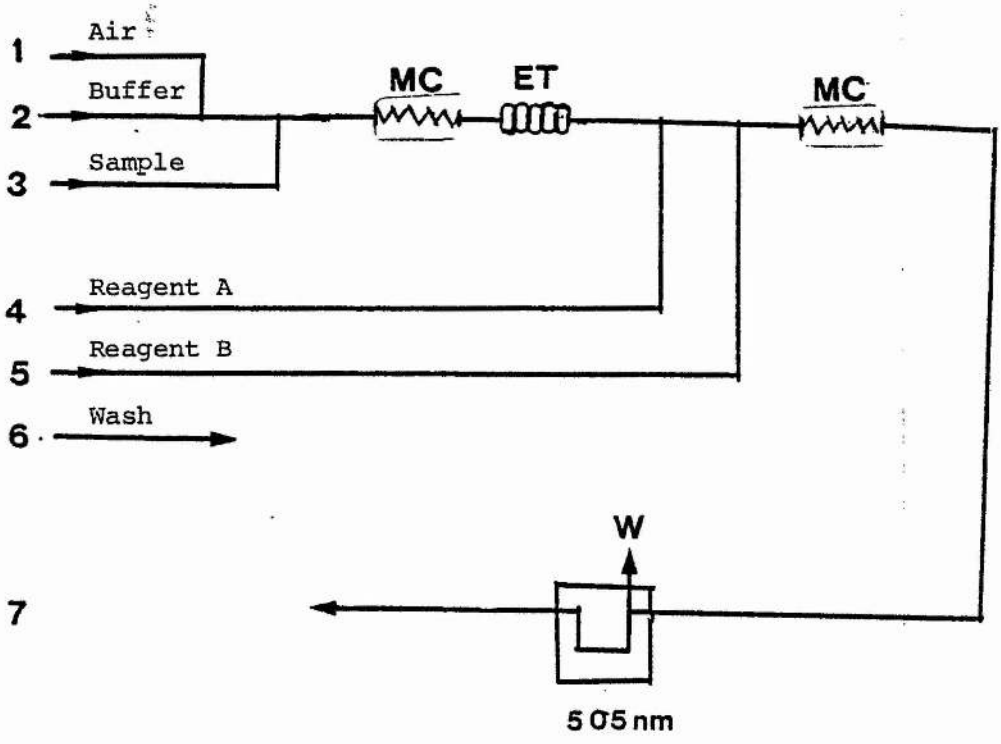


Fig. 5.1.2.1.1 Flow system for assaying the activity of immobilised GOD tube. The system consists of standard Technicon AutoAnalyser AA1 modules. The mixing coils (MC) and the enzyme tube (ET) are maintained at 37°. (W) indicates waste line.

Keys to Fig. 5.1.2.1.1

Pumping line		Flow rate ($ml.min^{-1}$)
1.	Air	0.8
2.	Buffer	1.6
3.	Sample	0.16
4.	Colour reagent A	0.23
5.	Colour reagent B	0.23
6.	Wash line; buffer	1.0
7.	Flow cell	1.6

Sampling rate: 60 $samples.h^{-1}$.

Sample to wash ratio: 2:1

Buffers used:

citrate	pH 3.4 - 5.5
acetate	4.5 - 5.5
phosphate	6.5 - 7.6
borate	8.5 - 10.0

5.1.2.2 Variation in PEI concentration

PEI was diluted in water and used as a spacer molecule between the nylon backbone and the enzyme molecule.

Procedure

Nylon tube was activated by 18.5%(w/v) glutaraldehyde in 0.5M borate buffer pH 9.0, and incubated at 90° for 15 min.

PEI was diluted to 1-10(w/v) in water. Activated nylon tube was filled with the diluted PEI solution and incubated at room temperature for 2h.

The tube was washed with a large volume of water to remove excess unbound PEI, and GOD was immobilised as described in section 5.1.1.4.

5.1.3 Preparation of nylon immobilised uricase

Reagents

1. Uricase; purified uricase was obtained by the purification procedure described in section 4.2.2. Uricase in borate buffer was titrated to pH 8.5-9.0 for coupling to activated tube.

Procedure

Nylon tube was activated directly with glutaraldehyde. Typically 2m nylon tube was wound around a perspex cylinder and filled with 18.5%(w/v) glutaraldehyde in 0.5M borate buffer pH 9.0. The tube was sealed end to end and incubated at 90° for 15 min.

The tube was washed with 0.5M NaCl solution and filled with 10%(w/v) PEI solution in water. The tube was incubated at room temperature for 2h.

The derivatised nylon tube was reactivated by either glutaraldehyde or bisimidate as described in section 5.1.1.5, before coupling of enzyme.

Reactivated nylon tube was filled with the enzyme solution and incubated for 3h at 4°. After coupling the tube was washed with 50mM glycine pH 9.0 containing 0.2M NaCl.

5.1.4 Assay of immobilised enzyme

5.1.4.1 Theory

The activity of a tubular enzyme reactor can be assayed by the recycling method described by Ford et al.(130). This method simulates soluble assay conditions and is preferred by many workers. Activity of an enzyme tube can be calculated by the formula;

$$v = -\frac{ds}{dt}\frac{(TV)}{L}$$

where,

v is the activity of enzyme derivative (μmole.min.$^{-1}$m^{-1}).

$-\frac{ds}{dt}$ is the observed rate of change in the substrate concentration.

TV is the total volume of assay (ml).

L is the length of enzyme tube (m).

Noy (92) adopted this method to assay alcohol, aldehyde and lactic dehydrogenase (lactic dehydrogenase; L-lactate: NAD oxidoreductase, EC 1.1.1.27) immobilised to nylon tube. These enzyme-catalysed reactions which use or liberate NADH, can be monitored at 340nm in a flow through cell.

In the case of uricase the reaction products are not easily monitored in this kind of system. A method involving the integrated form of Michaelis-Menten equation using single pass technique is more adaptable.

Two approaches are taken in using this method.

a. For changing urate concentration, zero order kinetics was assumed with respect to oxygen.

The Michaelis-Menten equation is given by:

$$-\frac{ds}{dt} = \frac{Vs}{s + K_m} = \frac{V}{1 + K_m/s} \qquad 1$$

where s - substrate concentration

V - maximum velocity

K_m - Michaelis constant

Thus,

$$-\int_{S_o}^{S_t} ds \qquad -K_m\int_{S_o}^{S_t}\frac{ds}{s} \qquad = V\int_{o}^{t} dt$$

$$-(S_t - S_o) - K_m \ln\frac{(S_t)}{(S_o)} = Vt \qquad 2$$

where,

S_o - initial substrate concentration

S_t - substrate concentration at time t.

If $F = \frac{S_o - S_t}{S_o}$

Equation 2 becomes,

$$FS_o - K_m \ln(1 - F) = Vt \qquad 3$$

$$V = K_s(E) \qquad 4$$

where,

K_s - rate constant (μmole.min.$^{-1}$ mg.$^{-1}$ enzyme)

(E) - concentration of enzyme (mg.ml^{-1})

In a tubular reactor,

$$t = \frac{\pi r^2 L}{Q} \qquad 5$$

where,

L - length of tube (m)

Q - flow rate of substrate through tube ($ml.min^{-1}$)

r - radius of tube (m)

t - residence time of substrate in the tube, assuming piston flow (min.).

Now if,

E_a - surface concentration of enzyme ($mg.m^{-2}$).

Total amount of enzyme in the tube;

$$= 2\pi r L E_a$$

Volume of tube = $\pi r^2 L$

$$\text{i.e. } (E) = \frac{2\pi r L E_a}{r^2 L} = \frac{2E_a\pi}{r} \qquad 6$$

If k_t' is the apparent activity of the tube per unit length ($\mu mole.min.^{-1} m^{-1}$), then,

Total Activity;

$$k_t' L = K_s 2\pi r L E_a$$

$$K_s = \frac{k_t'}{2\pi r E_a} \qquad 7$$

Therefore, substituting $v = K_s(E)$ with equations 5, 6 and 7,

$$Vt = \frac{k_t'}{2\pi r E_a} \cdot \frac{2E_a\pi}{r} \cdot \frac{r^2 L}{Q}$$

$$Vt = \frac{k_t' L}{Q} \qquad 8$$

Substituting equation 8 into equation 3,

$$FS_o - K_m' \ln(1 - F) = k_t' L Q^{-1} \qquad 9$$

where K_m' - apparent Michaelis constant for the immobilised enzyme.

Rearranging equation 9,

$$FS_o = K_m' \ln(1 - F) + k_t' L Q^{-1} \qquad 10$$

If values of F are measured at different initial concentration S_o at constant flow rate Q, then FS_o plotted against $\ln(1 - F)$ will give a straight line if K_m' and k_t' are constant at that flow rate.

The slope of the line will be equal to K_m' and the intercept on the FS_o axis will be equal to $k_t' L Q^{-1}$.

b. When only the maximum activity of the immobilised enzyme (k_t') is required, a more simplified approach is taken.

The rate of uricase catalysed reaction is given by,

$$v = \frac{V}{\frac{K_u}{(U)} + \frac{K_{O_2}}{(O_2)} + 1} \qquad 1$$

where,

v - the rate of reaction

V - maximum rate of reaction

K_u - the Michaelis constant for urate

(U) - the concentration of urate

K_{O2} - the Michaelis constant for oxygen

(O_2) - the concentration of oxygen

If the urate concentration is kept high with respect to K_u, then

$$\frac{K_u}{(U)} << 1$$

Equation 1 is approximated to

$$v = \frac{V}{1 + \frac{K_{O_2}}{(O_2)}} \qquad 2$$

(O_2) in air saturated water is 0.23mM.

i.e.,

$$- \frac{d(O_2)}{dt} = \frac{V}{1 + \frac{K_{O_2}}{(O_2)}} \qquad 3$$

Integrating equation 3,

$$\left[(O_2)_o - (O_2)_t\right] - K_{O_2} \ln \left[\frac{(O_2)_t}{(O_2)_o}\right] = Vt \qquad 4$$

$$\frac{(O_2)_o - (O_2)_t}{(O_2)_o} = F, \text{ the fractional conversion.}$$

Equation 4 becomes,

$$F(O_2)_o - K_{O_2} \ln(1 - F) = Vt \qquad 5$$

The series,

$$\ln(1 + x) = x - 1/2x^2 + 1/3x^3 - 1/4x^4 \ldots\ldots\ldots\ldots\ldots\ldots$$

thus,

$$\ln(1 - F) = -F - 1/2F^2 - 1/3F^3 - 1/4x^4 \ldots\ldots\ldots\ldots\ldots\ldots$$

If $F << 1$, say $F < 0.15$,

$\ln(1 - F)$ can be approximated to $-F$

Equation 5 becomes,

$$F(O_2)_o + K_{O_2}F = Vt \qquad 6$$

$$F = \frac{Vt}{(O_2)_o + K_{O_2}}$$

$$\frac{F}{t} = \frac{V}{(O_2)_o + K_{O_2}} = v \qquad 7$$

The fraction of substrate to produce conversion, F, is measured by the hydrogen peroxide produced in the reaction.

$$F = \frac{(H_2O_2)}{230}$$

(H_2O_2) - $\mu mole.ml^{-1}$

$$t = \frac{TV}{Q} \qquad 8$$

where,

t - time of residence of assay mixture in the tube (min).

Therefore, substituting t in equation 7,

$$v = \frac{FQ}{TV} \qquad 9$$

Thus, the activity of the enzyme tube can be determined if the fraction of substrate consumed in the reaction, the flow rate of substrate through the enzyme tube and the volume of substrate assayed are measured.

Hydrogen peroxide produced in uricase catalysed reaction was determined by a coupling reaction involving two oxidative reactants, 4-aminophenazone(APZ) and dichlorohydroxy benzoyl sulphonate, catalysed by peroxidase (POD).

5.1.4.2 Preparation of dichlorohydroxy benzoyl sulphonate (HBS)

Procedure

3,5-dichloro-2-hydroxybenzene sulphonyl chloride (100 g) was gradually added to 1.5ℓ of distilled water with constant stirring. Sodium carbonate (40 g) was added to the mixture and the stirring continued for 2h at room temperature.

All undissolved materials were removed by vacuum filtration on a sintered glass funnel. The filtrate was evaporated on a rotary evaporator at 40°.

The white powder produced was dried over air and stored at room temperature.

5.1.4.3 Methods for assaying immobilised enzyme

Reagents

1. Colour reagents

A. HBS (3.5 $mg.ml^{-1}$) in 0.2M phosphate buffer pH 7.0. (17mM)

B. APZ (0.3 $mg.ml^{-1}$) in phosphate buffer pH 7.0, (1.5mM), and 0.02 $mg.ml^{-1}$ POD in 0.2M phosphate pH 7.0.

2. Hydrogen peroxide standard

A stock solution of about 100mM was stored in a dark bottle at 4°. Prior to use, the concentration of H_2O_2 was determined with acidified solutions of potassium permanganate. Working solutions were prepared by dilution in ice-cold water.

a. Immobilised GOD

3. Buffer; 0.1M phosphate buffer pH 7.0, containing 0.1M Na_2SO_4.

4. Substrate; 0.5M D-glucose in buffer.

b. Immobilised uricase

5. Buffer; 50mM borate buffer, 0.1M NaCl pH 8.6.

6. Substrate: 2mM uric acid was dissolved in the above buffer and diluted accordingly.

Procedure

A suitable length of immobilised enzyme tube (5 - 20 cm) was incorporated into the flow system shown in Fig. 5.1.4.3.1. Unless otherwise specified, the substrate flow through the system was air-segmented. Substrate was pumped through the enzyme tube at a certain flow rate, Q $ml.min^{-1}$ and 5 ml aliquots of effluent were collected.

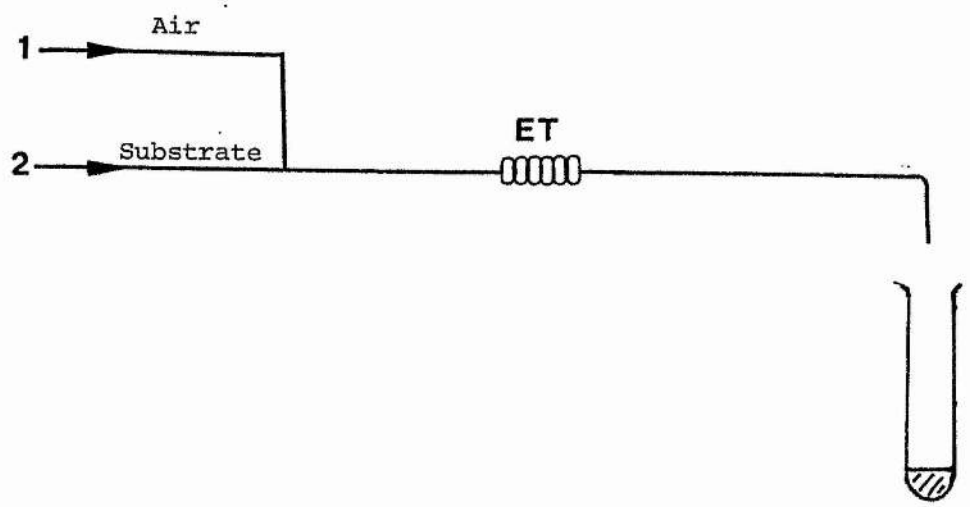


Fig. 5.1.4.3.1 Flow system for assaying immobilised enzyme (ET).

1 ml of reagent A and 1 ml of reagent B were added to the effluent and incubated at 25° for 15 min. Absorbance was read at 517nm.

A calibration plot was obtained by assaying standard solutions of hydrogen peroxide (Fig. 5.1.4.3.2).

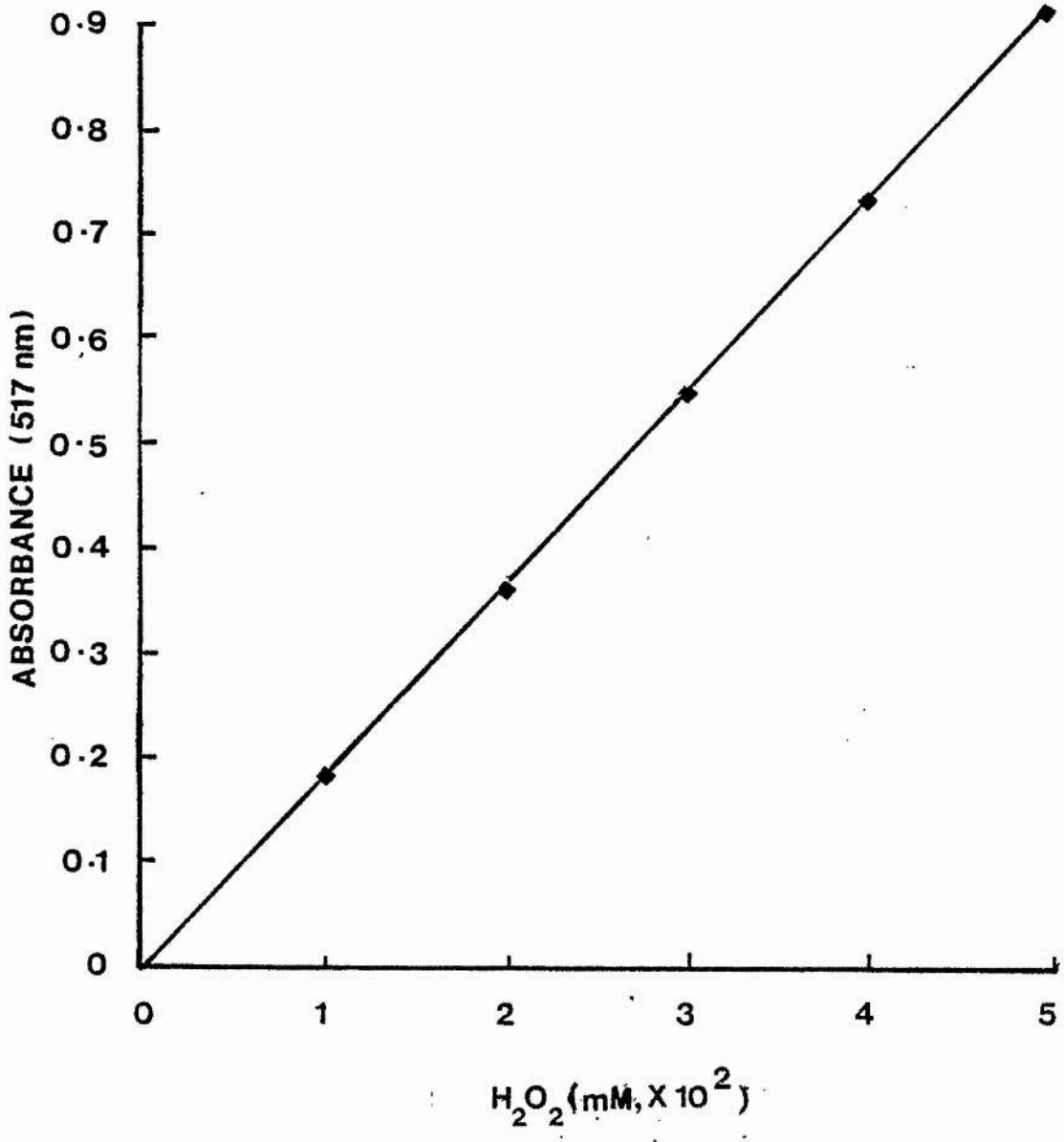


Fig. 5.1.4.3.2 Calibration plot for determination of hydrogen peroxide.

5.2 Results

5.2.1 Optimisation of glutaraldehyde activation of nylon tube

The optimal conditions for activation of nylon tube were studied. Glucose oxidase (GOD) was used as an index of the capacity of the activated nylon tube for enzyme retention on immobilisation. PEI was used as the spacer (section 5.1.2) and glutaraldehyde was used as the coupling reagent for enzyme immobilisation (section 5.1.1.4). The activity of each enzyme tube was assayed as described in section 5.1.2, and was expressed as a percentage of maximum tube activity obtained, i.e. as a relative activity.

Nylon tube treated with glutaraldehyde solution turned yellow in colour. Increased concentration of glutaraldehyde produced enzyme tubes with increased activity retention. Fig. 5.2.1.1 showed the effect of increasing glutaraldehyde concentration with activity retention. A maximum activity was achieved at 18.5%(w/v) glutaraldehyde solution. Further increase in glutaraldehyde concentration did not effectively increase activity retention, but the nylon tube turned brown in colour, and there was very obvious interaction between samples when the enzyme tube was assayed (see section 6.1.2.5 for explanation of the sample interaction effect).

Increasing period of incubation at 90° increased activity retention. Fig. 5.2.1.2 illustrates the results obtained showing a maximum activity retention at 15 min incubation period.

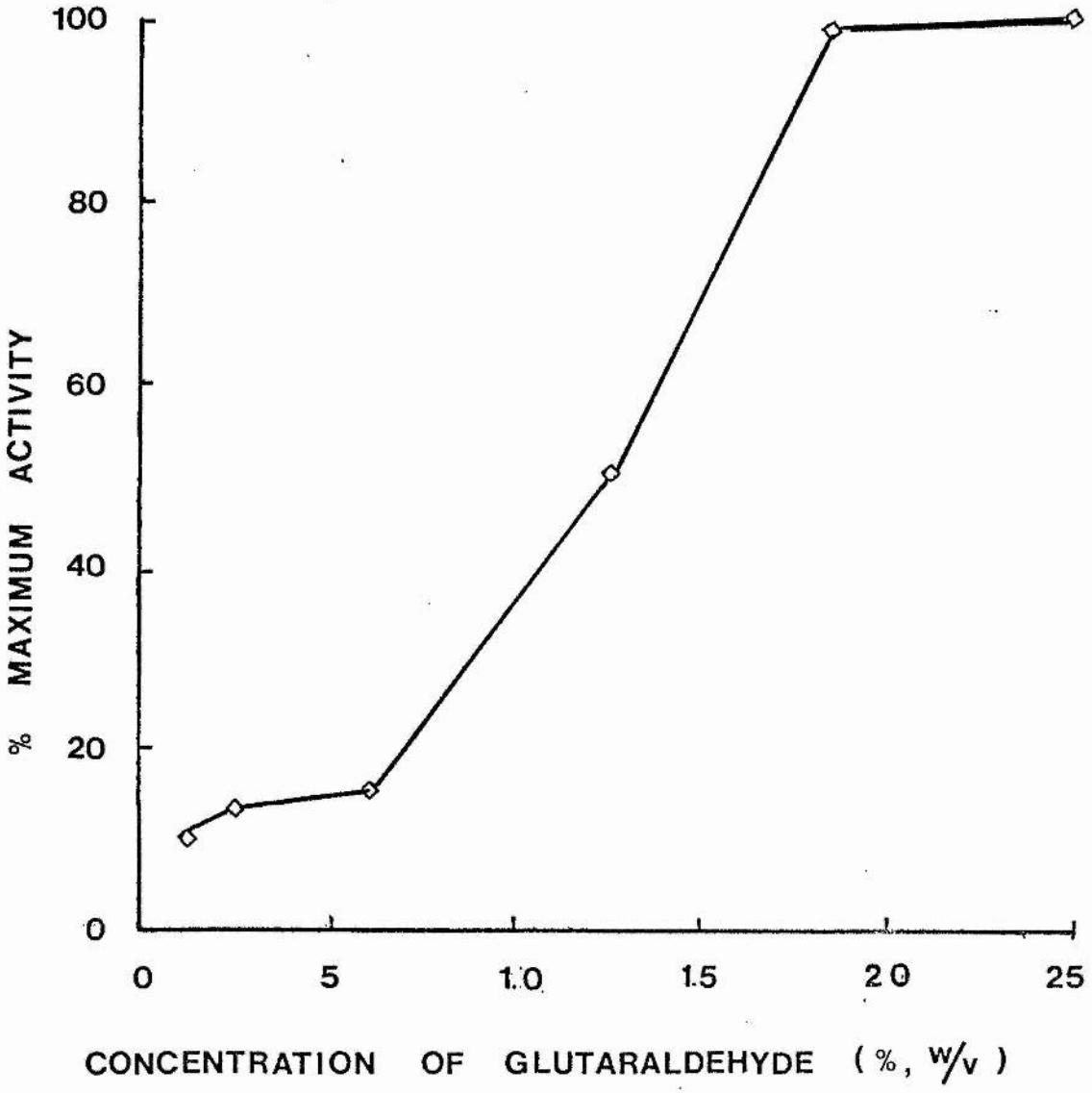


Fig. 5.2.1.1 Effect of glutaraldehyde concentration on activity retention.

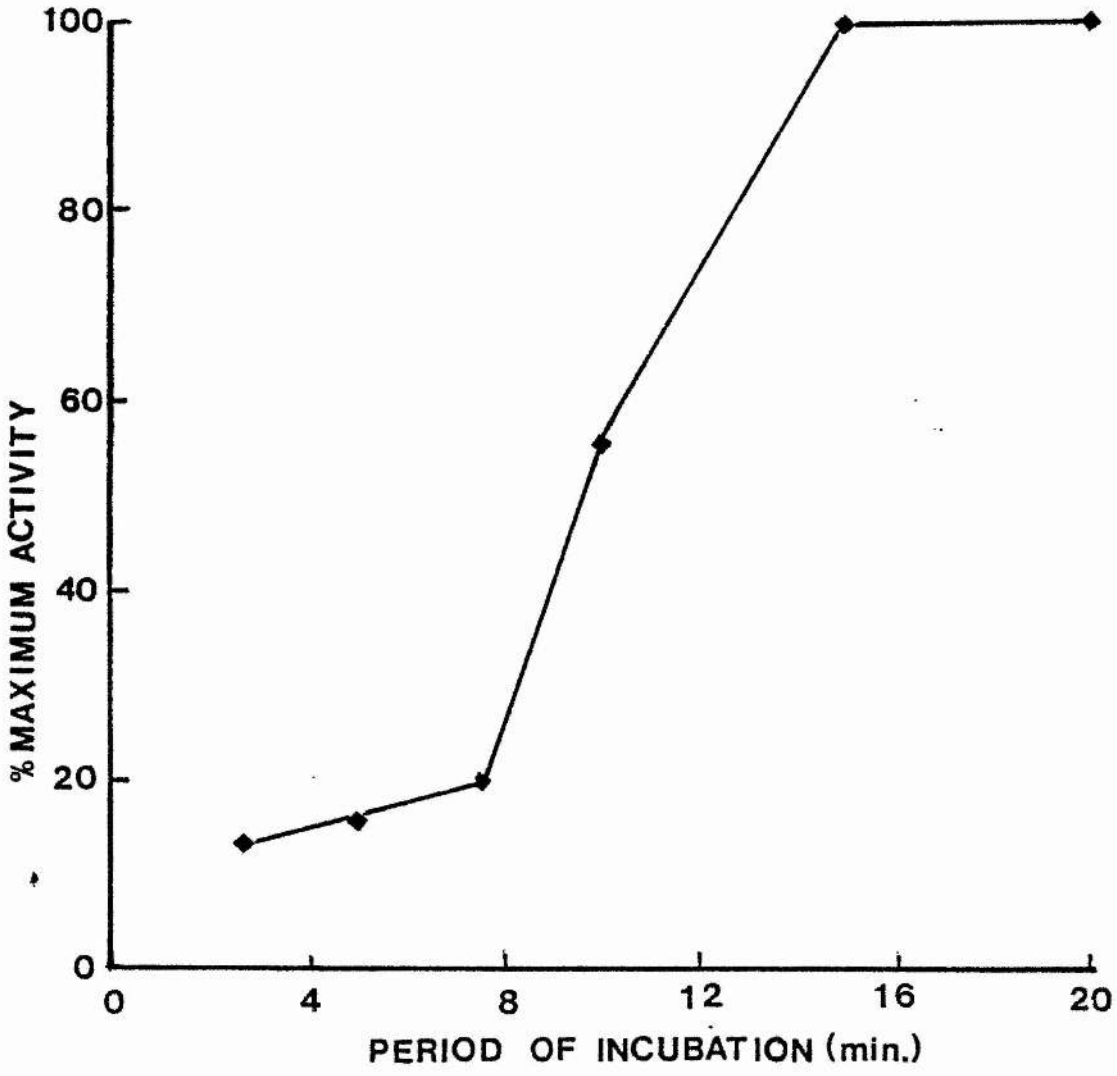


Fig. 5.2.1.2 Effect of period of incubation on activity retention.

Longer period of exposure to glutaraldehyde produced brown coloured tube with no further increase in activity retention.

Maximum activity retention was achieved when nylon tube was incubated at 90°, the maximum temperature studied. The relative activity retention at varying temperature is shown in Fig. 5.2.1.3.

Fig. 5.2.1.4 shows the effect of pH of glutaraldehyde solution in the activation of nylon tube. There seemed to be high activation of nylon at both acidic and alkaline pH values. High activity enzyme tubes were produced when nylon was activated by glutaraldehyde in these pH values. However tubes activated by acidic glutaraldehyde solution resulted in brown colouration with obvious high sample interaction during continuous analysis of samples.

In all conditions studied, protein coupling increased with activity retention, but as the absolute concentration of protein was not determined, the effect with respect to specific activity of enzyme coupled to the activated tube was not presented.

It is worthwhile noting that in assaying the activity of enzyme tubes with high sample interactions, the activity observed may be higher than it should be. The effect of preceeding sample analysed would falsely elevate the subsequent sample above its true value.

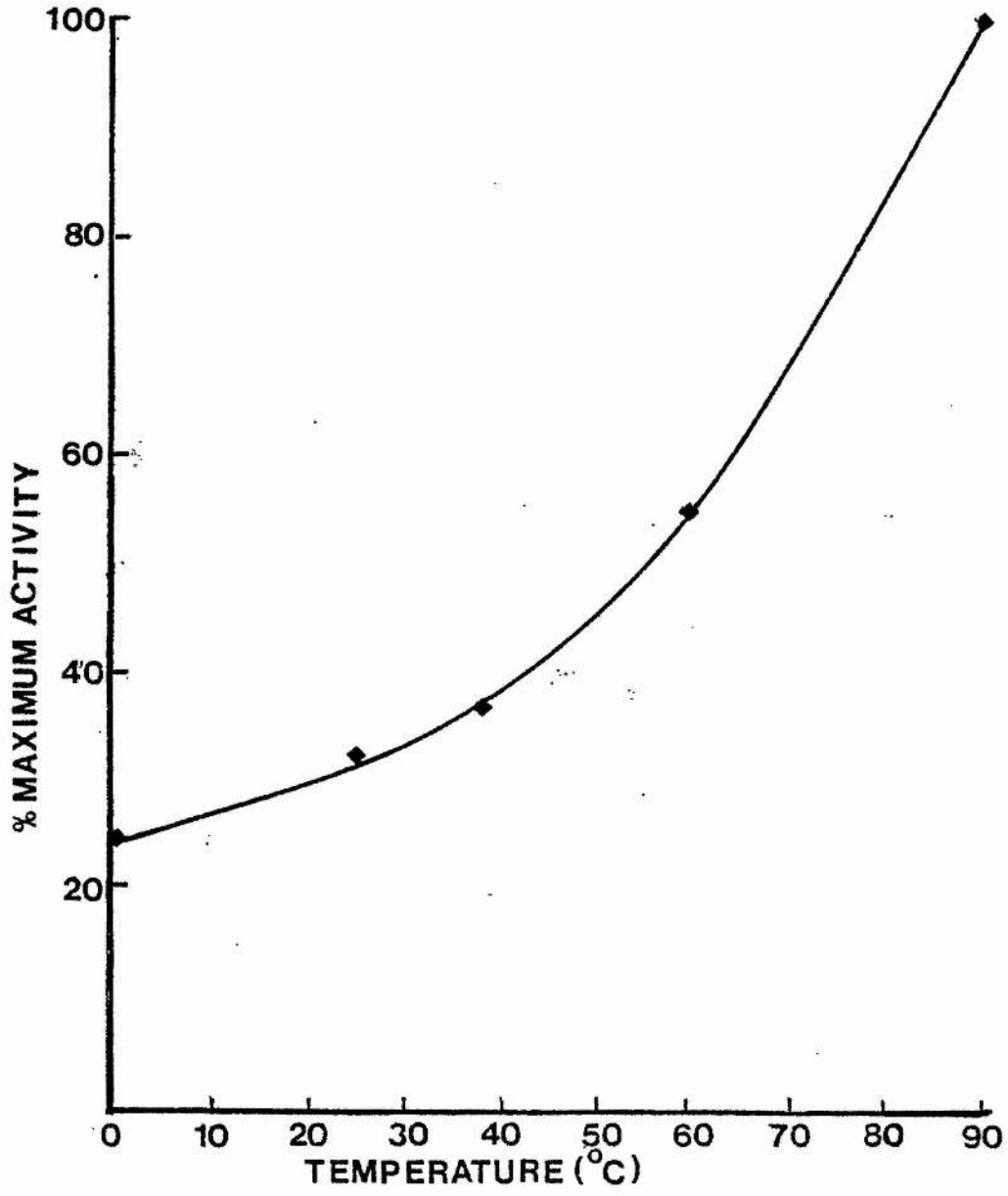


Fig. 5.2.1.3 Effect of temperature of activation on activity retention.

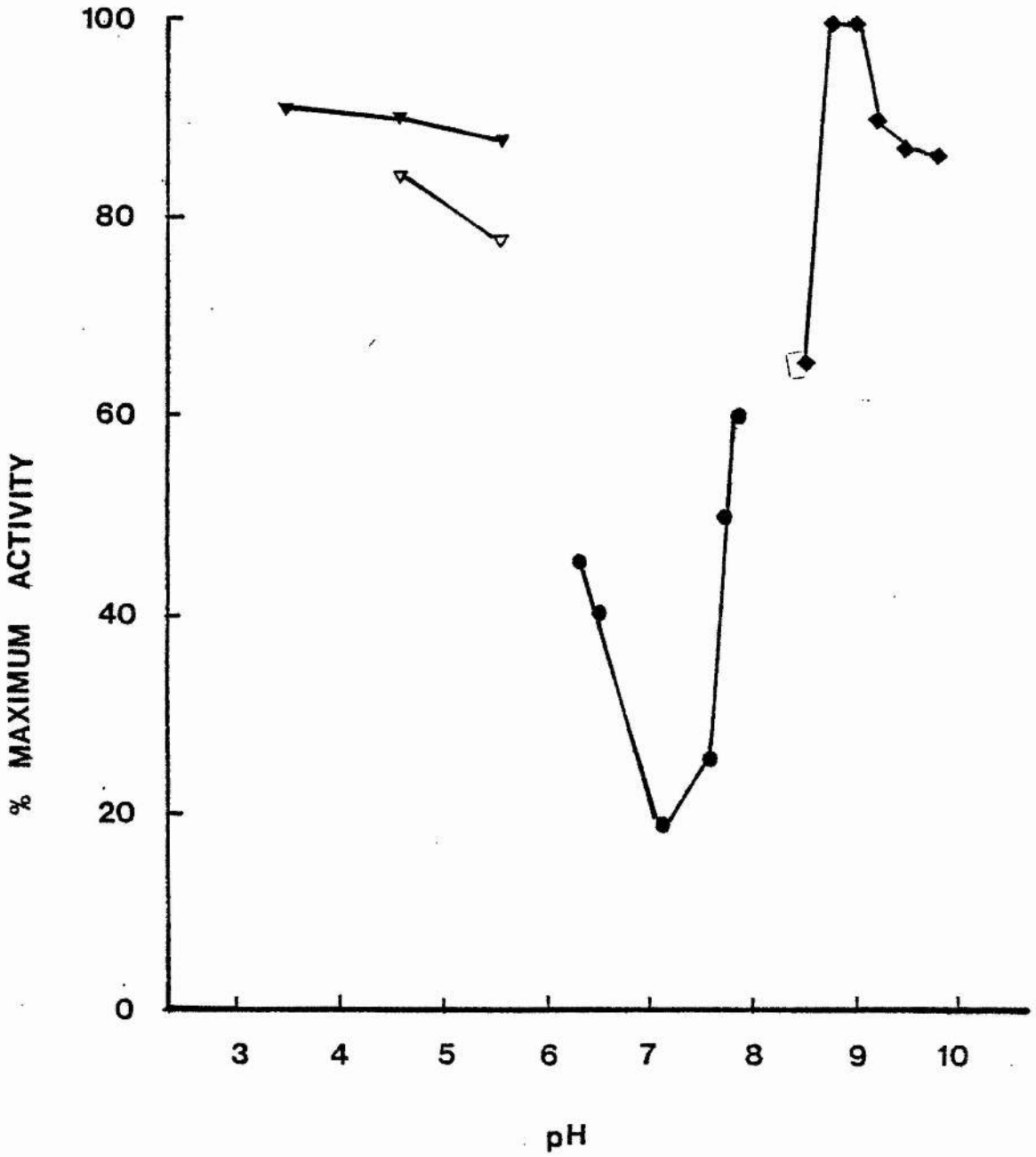


Fig. 5.2.1.4 Effect of pH of glutaraldehyde solutions in the activation of nylon tube. Glutaraldehyde was diluted in citrate (▼), acetate (▽), phosphate (●) and borate (◆) buffers.

5.2.2 Optimum PEI concentration for spacer coupling to activated nylon tube

Nylon tube was O-alkylated as described in section 5.1.1.2 and substituted with PEI, adipic hydrazide and diaminoethane (section 5.1.1.3). GOD was coupled by glutaraldehyde activation (section 5.1.1.4). Generally it was found that enzyme tube with adipic hydrazide and diaminoethane spacers retained about 80% activity retained by enzyme tubes with PEI as spacer.

Nylon tube was activated by 18.5%(w/v) glutaraldehyde at 90° for 15 min, and varying concentrations of PEI were used for the coupling of spacer to the activated tube (section 5.1.1.2). Fig. 5.2.2.1 shows the relative enzyme activity retention of these tubes on GOD immobilisation. 10%(w/v) PEI solution shows maximum activity retention, this PEI concentration being the maximum concentration investigated. Higher concentration of PEI coagulated inside the nylon tubes during the coupling period (spacer attachment) and there was difficulty in removal of excess unbound PEI.

5.2.3 Comparison between immobilised enzyme derivatives prepared from O-alkylated and glutaraldehyde activated nylon tube

Nylon tube was either O-alkylated (section 5.1.1.2) or directly activated by glutaraldehyde (section 5.1.3). The activated nylon tube was attached with PEI as spacer (section 5.1.1, 5.1.3) and GOD was immobilised to the tube after reactivation by glutaraldehyde (section 5.1.1.4).

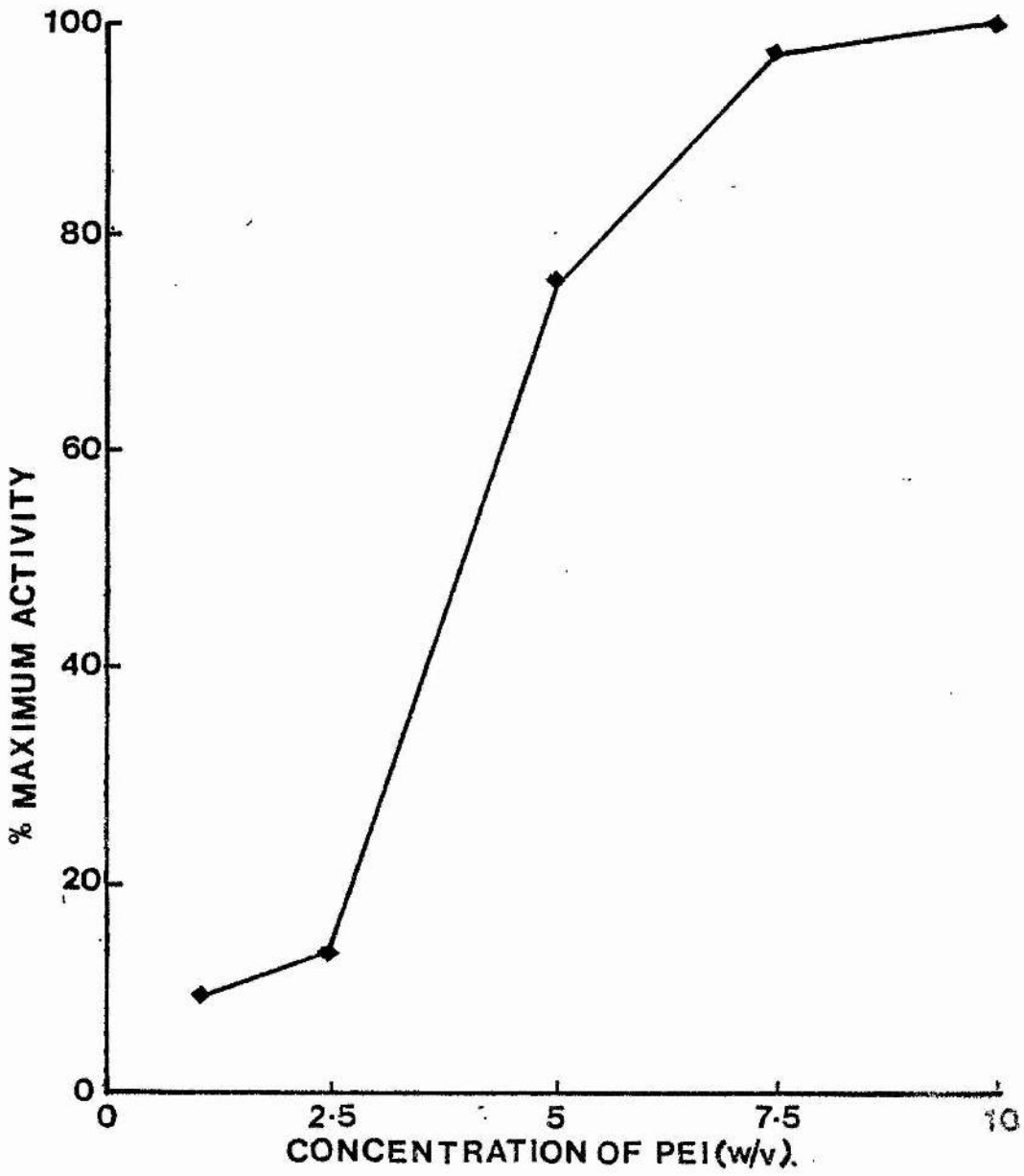


Fig. 5.2.2.1 Effect of PEI concentration on activity retention.

Table 5.2.3.1 summarises the result obtained. O-alkylated enzyme tube has about 70% of the activity retained by glutaraldehyde activated nylon tube.

5.2.4 Comparison between glutaraldehyde and bisimidate coupling of enzyme to PEI-derivatised nylon tube

Table 5.2.4.1 summarises the result obtained when GOD was coupled by glutaraldehyde or bismidate (section 5.1.1.4). Nylon tube was activated by glutaraldehyde and attached with PEI as spacer (section 5.1.3).

The results show no significant differences in the amount of activity and protein retained by either of these methods.

5.2.5 Immobilisation of uricase to nylon tube

5.2.5.1 Immobilisation of uricase to O-alkylated and glutaraldehyde activated nylon tube

Nylon tube was O-alkylated (section 5.1.1.2) and activated by glutaraldehyde (section 5.1.3) and the activated tube was coupled to PEI (section 5.1.1.3, 5.1.4) and uricase was immobilised as described in section 5.1.3.

Table 5.2.5.1.1 summarises the result obtained which shows that a higher activity enzyme tube is produced with glutaraldehyde activation. About twice the activity was retained by glutaraldehyde activated tube as there was in TOTFB-activated nylon tube.

Method of Activation	Pre-coupling solution		Post-coupling solution		k_t' ($U.m^{-1}$)	% retention	
	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)		Activity	Protein
Glutaraldehyde activation	66	0.8	3.4	0.036	21.6	22	95
TOTFB activation	66	0.8	10.0	0.25	15.8	16	69

Table 5.2.3.1 Comparison between O-alkylated and glutaraldehyde activated nylon tubes. Activity of immobilised GOD was assayed as described in section 5.1.4.3, without air-segmentation of substrate flow, at a flow rate of 60 $ml.min^{-1}$. k_t' was calculated by the formula $k_t'L = \frac{FQ}{TV}$ (section 5.1.4.1b).

Coupling agent	Pre-coupling solution		Post-coupling solution		k_t' ($U.m^{-1}$)	% retention	
	Activity ($u.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)		Activity	Protein
Glutaraldehyde	66	0.8	4.0	0.05	20.0	20.0	94
Bisimidate	66	0.8	4.0	0.04	18.2	18.4	95

Table 5.2.4.1 Comparison between glutaraldehyde and bisimidate reactivation of PEI-derivatised nylon tube. Activity of immobilised GOD was assayed as described in section 5.1.4.3 without air-segmentation of substrate flow, at a flow rate of 60 $ml.min^{-1}$. $k_t'L = \frac{FQ}{TV}$ (section 5.1.4.1b)

Nylon derivatives	Pre-coupling solution		Post-coupling solution		k_t' ($U.m^{-1}$)	% retention	
	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)		Activity	Protein
PEI-Glutaraldehyde -nylon	1.3	0.2	0.05	0.02	0.28	17	75
PEI-TOTFB-nylon	1.3	0.2	0.11	0.15	0.14	8	45

Table 5.2.5.1.1 Immobilisation of uricase to O-alkylated and glutaraldehyde-activated nylon tube. Immobilised uricase was assayed by the method described in section 5.1.4.3, where $k_t'L = \frac{FQ}{TV}$. For the flow rate used see Fig. 5.2.5.5.4.

5.2.5.2 Immobilisation of uricase to derivatised nylon tube by glutaraldehyde and bisimidate coupling

PEI-derivatised nylon tubes were prepared as described in section 5.1.3. Glutaraldehyde and bisimidate coupling were as described in section 5.1.1.5. Fig. 5.2.5.2.1 summarises the data obtained, which shows no particular variation between the two coupling agents in the immobilisation of uricase.

5.2.5.3 Immobilisation of uricase to nylon tube using different concentrations of uricase in the coupling solution

PEI-derivatised nylon was prepared as described in section 5.1.3. Different concentrations of uricase (in coupling solutions) were coupled to the tube by glutaraldehyde (section 5.1.1.4). A commercial preparation of uricase from *Candida utilis* was also immobilised as above.

Table 5.2.5.3.1 summarises the results obtained. The enzyme tube activity (k_t') increased with higher concentration of uricase in the coupling solution. Activity retention increased with increased concentration of enzyme in coupling solution, but it is significant that at the highest concentration investigated uricase activity could be detected in the post-coupling solution.

Coupling Agents	Pre-coupling solution		Post-coupling solution		k_t'	% retention	
	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	($U.m^{-1}$)	Activity	Protein
Glutaraldehyde	1.9	0.35	0.04	0.04	0.37	16.8	88
Dimethyl suberimidate	1.5	0.35	0.06	0.04	0.40	17.7	88

Table 5.2.5.2.1 Immobilisation of uricase to PEI-derivatised nylon tube using glutaraldehyde and bisimidate coupling. Immobilised uricase was assayed according to the method described in section 5.1.4.3, where $k_t'L = \frac{FQ}{TV}$.

Coupling solution		Post-coupling solution		k_t'	& Retention	
Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	($U.m^{-1}$)	Activity	Protein
1.3	0.36	0.05	0.02	0.39	20.5	95
0.85	0.18	0	0.015	0.23	18	92
0.5	0.14	0	0.015	0.11	11	89
1.5*	1.6	0.18	0.13	0.35	20	89

Table 5.2.5.3.1 Data obtained when different concentrations of uricase in the coupling solution were used in the immobilisation of uricase to PEI-derivatised nylon. (*) denoted enzymes from *Candida utilis*. k_t was calculated by the formula $k_t'L = \frac{FQ}{TV}$.

5.2.5.4 Immobilisation of uricase to nylon tube in the presence of substrate/inhibitor in the coupling solutions

PEI-derivatised nylon tube was prepared as described in section 5.1.3, and uricase was immobilised by glutaraldehyde coupling (section 5.1.1.4).

Table 5.2.5.4.1 summarises the results obtained with different coupling solutions. Evidently the presence of a substrate/inhibitor in the coupling solution improved the percentage activity retention. On the basis of the data available, the presence of substrate seemed to be more favourable for higher activity retention than that of the inhibitor.

5.2.5.5 Determination of K_m' and k_t' of immobilised uricase

Immobilised uricase was prepared as described in section 5.1.3, and assayed according to the method described in section 5.1.4.3. Substrate concentrations (urate) in the range of 0.025 to 1mM were used.

a. Effect of air segmentation

Fig. 5.2.5.5.1 shows the relationship between FS_o and $\ln(1 - F)$ in the presence and absence of air-segmentation in the substrate flow through the enzyme tube. Two buffers were used in the studies; i.e. 50mM borate, 0.1M NaCl pH 8.6, and 50mM glycine, 0.1M NaCl pH 9.0.

Coupling solution	Pre-coupling solution		Post-coupling solution		k_t' ($U.m^{-1}$)	% Retention	
	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Activity ($U.ml^{-1}$)	Protein ($mg.ml^{-1}$)		Activity	Protein
Uricase in buffer pH 8.5	1.2	0.25	0.04	0.04	0.36	20	84
Uricase in buffer with 1.5mM oxonate pH 8.5	1.14	0.20	-	0.04	0.23	40	80
Uricase in buffer with 1mM uric acid pH 8.5	0.98	0.18	-	0.035	0.30	61	81

Table 5.2.5.4.1 Data obtained on immobilisation of uricase to nylon tube, in the presence of uric acid and oxonate. Immobilised uricase was assayed as described in section 5.1.4.3, where $k_t'L = \frac{FQ}{TV}$.

Significantly, k_t' was higher in glycine buffer and when the uricase tube was assayed with air-segmentations in the substrate flow. The K_m' values were similar in all cases except when the immobilised enzyme was assayed in glycine buffer without air-segmentation in the substrate flow. Table 5.2.5.5.1 summarises the values of K_m' and k_t' obtained from the plot.

b. Effect of ionic concentration

Fig. 5.2.5.5.2 shows the relationship between FS_o and $\ln(1 - F)$ for different ionic buffer strengths. Linear plots were obtained with buffers containing NaCl. There was some variations in the values of k_t' obtained with different buffer systems used. Table 5.2.5.5.2 shows the values of k_t' and K_m' obtained from the plot.

c. Effect of flow rate

Fig. 5.2.5.5.3 shows the relationship between FS_o and $\ln(1 - F)$ with different flow rates of substrate through the enzyme tube. Table 5.2.5.5.3 shows the values of K_m' and k_t' obtained from this plot.

There seemed to be an increase in k_t' values with higher flow rates, but there appeared to be no variation in the values of K_m'.

The variation of k_t' with flow rate was also studied by calculating k_t' using the formula $k_t'L = \frac{FQ}{TV}$ (section 5.1.4.1b). A plot of percentage of oxygen consumption in relation to flow rate and k_t' is shown in Fig. 5.2.5.5.4,

Buffer systems	Without air-segmentation		With air-segmentation	
	K_m' (x 10^5M)	k_t' ($U.m^{-1}$)	K_m' (x 10^5M)	k_t' ($U.m^{-1}$)
50mM borate, 0.1M NaCl pH 8.6	4.1	0.24	4.3	0.34
50mM glycine, 0.1M NaCl pH 9.0	8.4	0.28	4.3	0.36

Table 5.2.5.5.1 K_m' and k_t' values obtained when assayed with and without air-segmentation in the substrate flow. These values were obtained from the graph shown in Fig. 5.2.5.5.1.

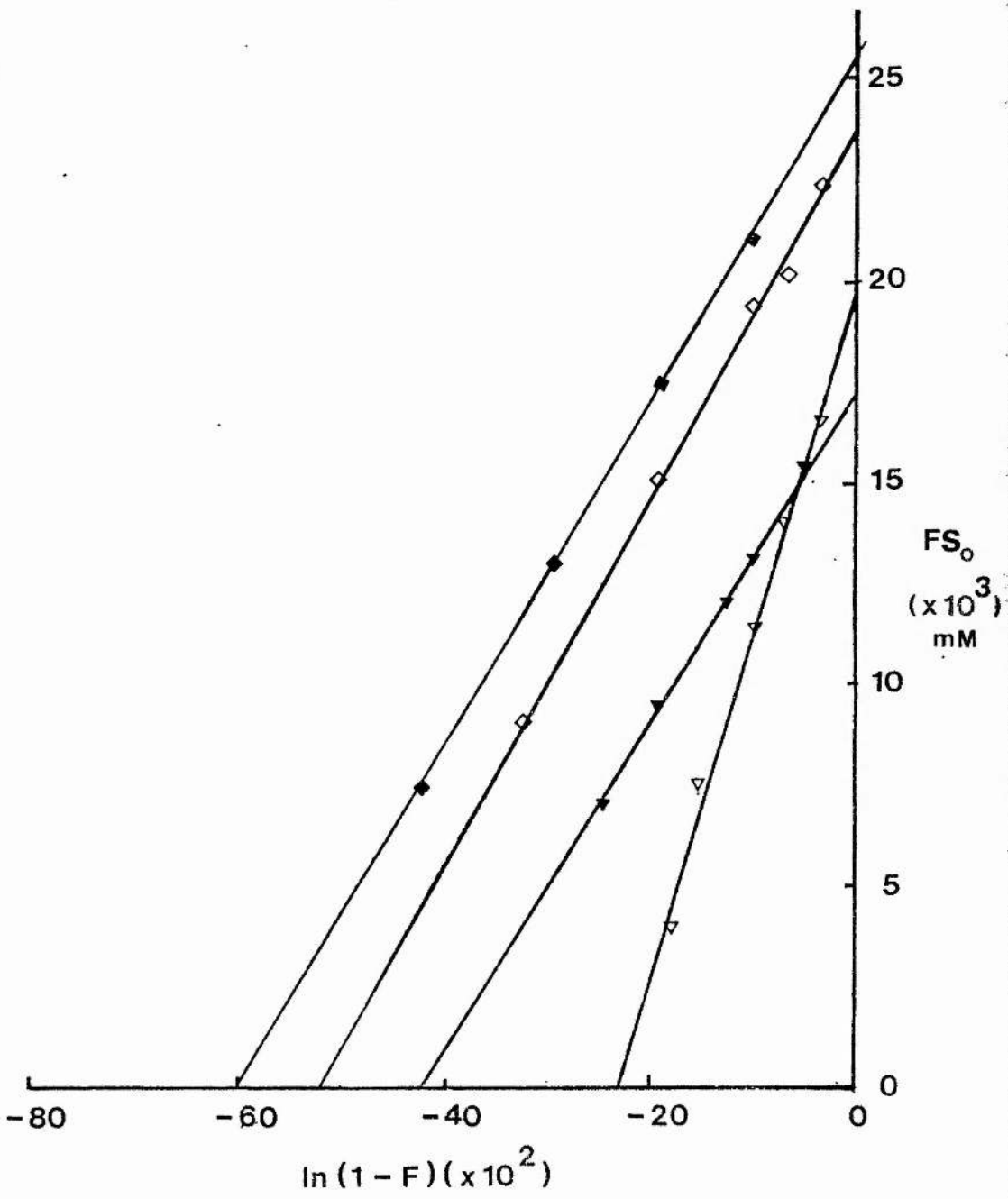


Fig. 5.2.5.5.1 Relationship between FS_o and ln(1 - F) with and without air segmentation in substrate flow during assay (section 5.2.5.5a). Flow rate was 1.4 $ml.min^{-1}$. Enzyme tube (10 cm) was assayed with urate in borate buffer pH 8.6, without air-segmentation (▼), with air segmentation (◇) and in glycine buffer pH 9.0 without air-segmentation (▽) and with air segmentation (◆).

Buffer systems	$K_{m'}$ (x 10^5M)	$k_{t'}$ ($U.m^{-1}$)
50mM glycine pH 9.0	-	0.28
50mM glycine pH 9.0 with 0.1M NaCl	4.1	0.36
50mM glycine pH 9.0 with 0.5M NaCl	5.8	0.34
50mM borate pH 8.6 with 0.1M NaCl	4.5	0.34

Table 5.2.5.5.2 Variation in $K_{m'}$ and $k_{t'}$ with ionic strengths. The values were calculated from the plots on Fig. 5.2.5.5.2.

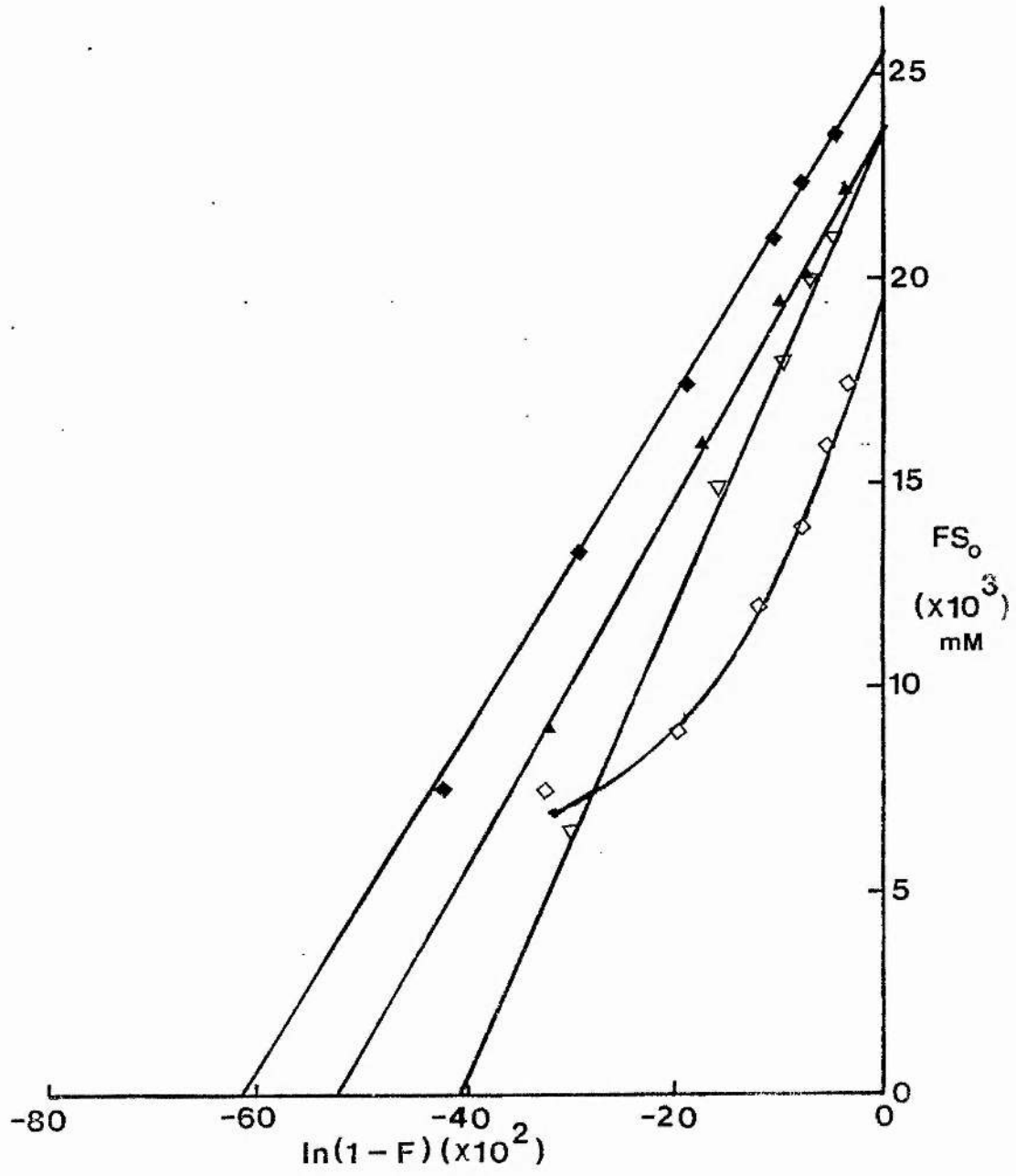


Fig. 5.2.5.5.2 Relationship between FS_o and ln(1 - F) with different ionic strengths. Enzyme tube (10 cm) was assayed with urate dissolved in the following buffers; 50mM glycine pH 9.0 (◇), 50mM glycine, 0.1M NaCl pH 9.0 (◆), 50 mM glycine (0.5M NaCl pH 9.0 (▽) and 50mM borate, 0.1M NaCl pH 8.6 (▲). Flow rate was 1.4 ml.min^{-1}.

Flow rate ($ml.min^{-}$)	K_m' ($\times 10^5 M$)	k_t' ($U.m^{-1}$)
1.4	3.8	0.37
2.0	3.9	0.40
2.4	4.0	0.43

Table 5.2.5.5.3 Variation in K_m' and k_t' values with different flow rates. The values were calculated from the plots of Fig. 5.2.5.5.3.

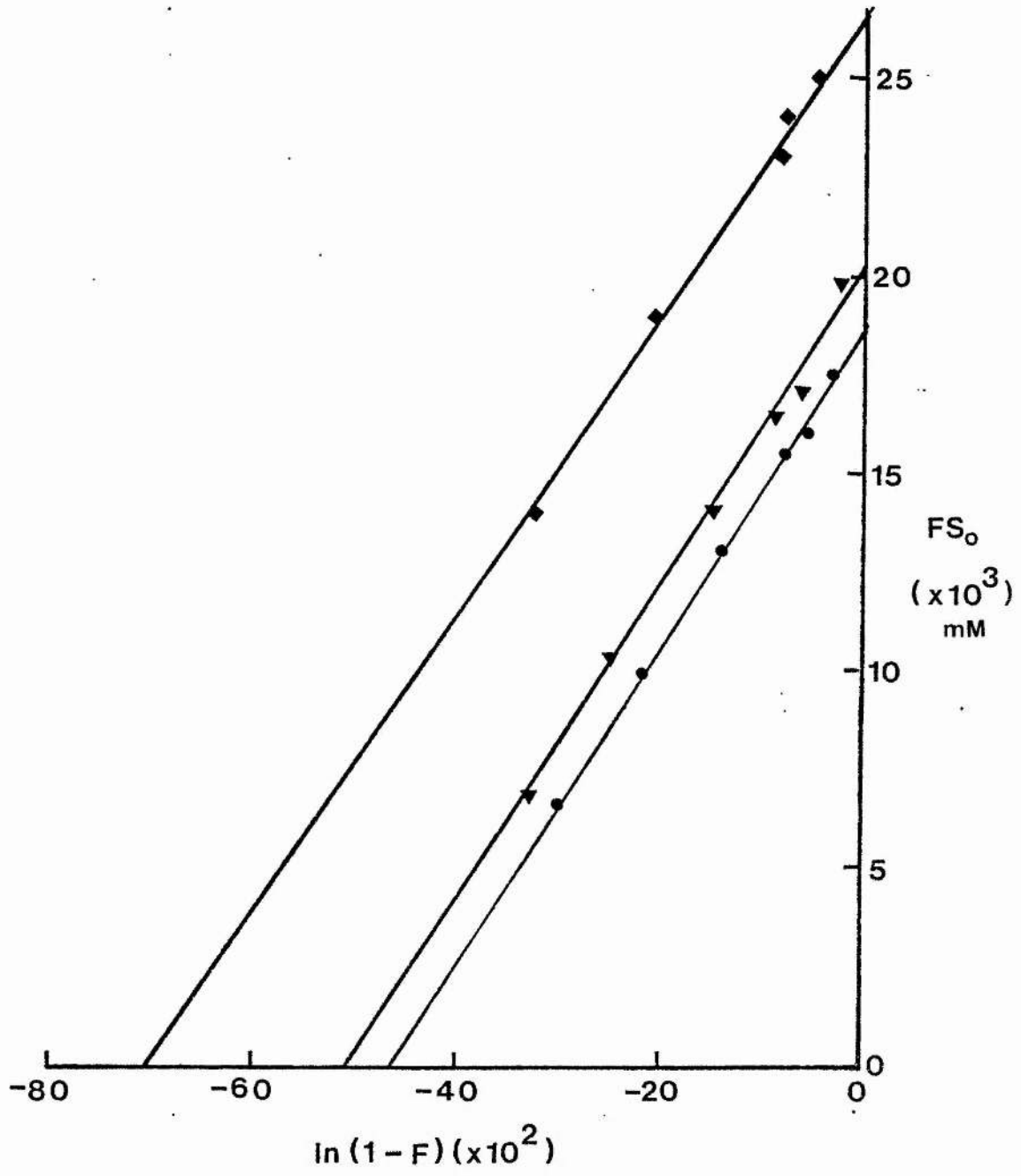


Fig. 5.2.5.5.3 Relationship between FS_o and ln(1 - F) with different flow rates. The flow was air-segmented and the substrate dissolved in 50mM glycine, 0.1M NaCl pH 9.0. The flow rate investigated was 1.4 ml.min^{-1} (◆), 2.0 ml.min^{-1} (▼) and 2.4 ml.min^{-1} (●).

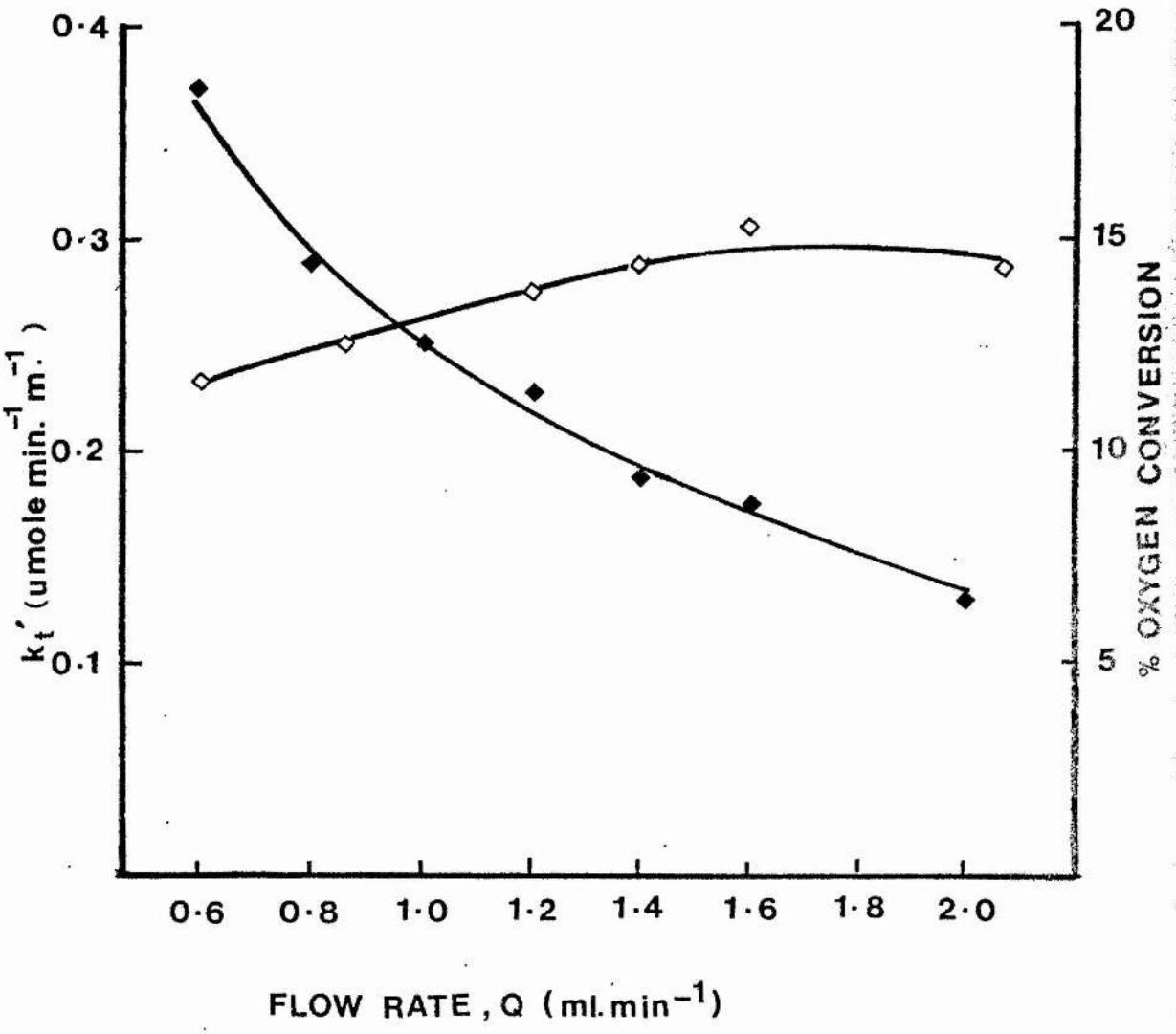


Fig. 5.2.5.5.4 k_t' in relation to the flow rates and percentage of oxygen conversion, where $k_t'L = \frac{FQ}{TV}$ (section 5.1.4.15). (◇) denoted k_t and (◆) denoted percentage oxygen conversion.

5.2.5.6 pH profile of immobilised uricase

Immobilised uricase was assayed in a range of pH values, and the tube activity (k_t') assayed (section 5.1.4.1b). The range of pH of 7.6 to 10.0 was studied, with 0.1M TRIS-HCl (0.1M with respect to NaCl), and 50mM borate (0.1M with respect to NaCl), used over the range of pH values. Fig. 5.2.5.6.1 shows the pH profile of immobilised uricase with the precentage maximum activity plotted against pH. Optimum pH for the immobilised uricase was observed to be in the range 9.0 - 9.2.

5.2.5.7 Thermal denaturation of immobilised uricase

Immobilised uricase tubes were prepared (section 5.1.3) and the activity assayed (section 5.1.4.3). k_t' was calculated according to section 5.1.4.1b.

Enzyme tubes (10 cm) were assayed for activity, and incubated in a series of buffer at 37° for set period of incubation. After the incubation period the tubes were immersed in ice-cold buffer, and then assayed for residual activity. Fig. 5.2.5.7.1 shows the residual activity plotted against incubation periods for different buffer systems. Significantly there was low thermal resistance by the immobilised uricase when the tube was incubated in borate buffer, and there appeared to be greater denaturation of enzyme when the tube was incubated in high pH buffer. The use of high ionic concentration in the buffer did not alter the denaturation characteristics of the immobilised uricase.

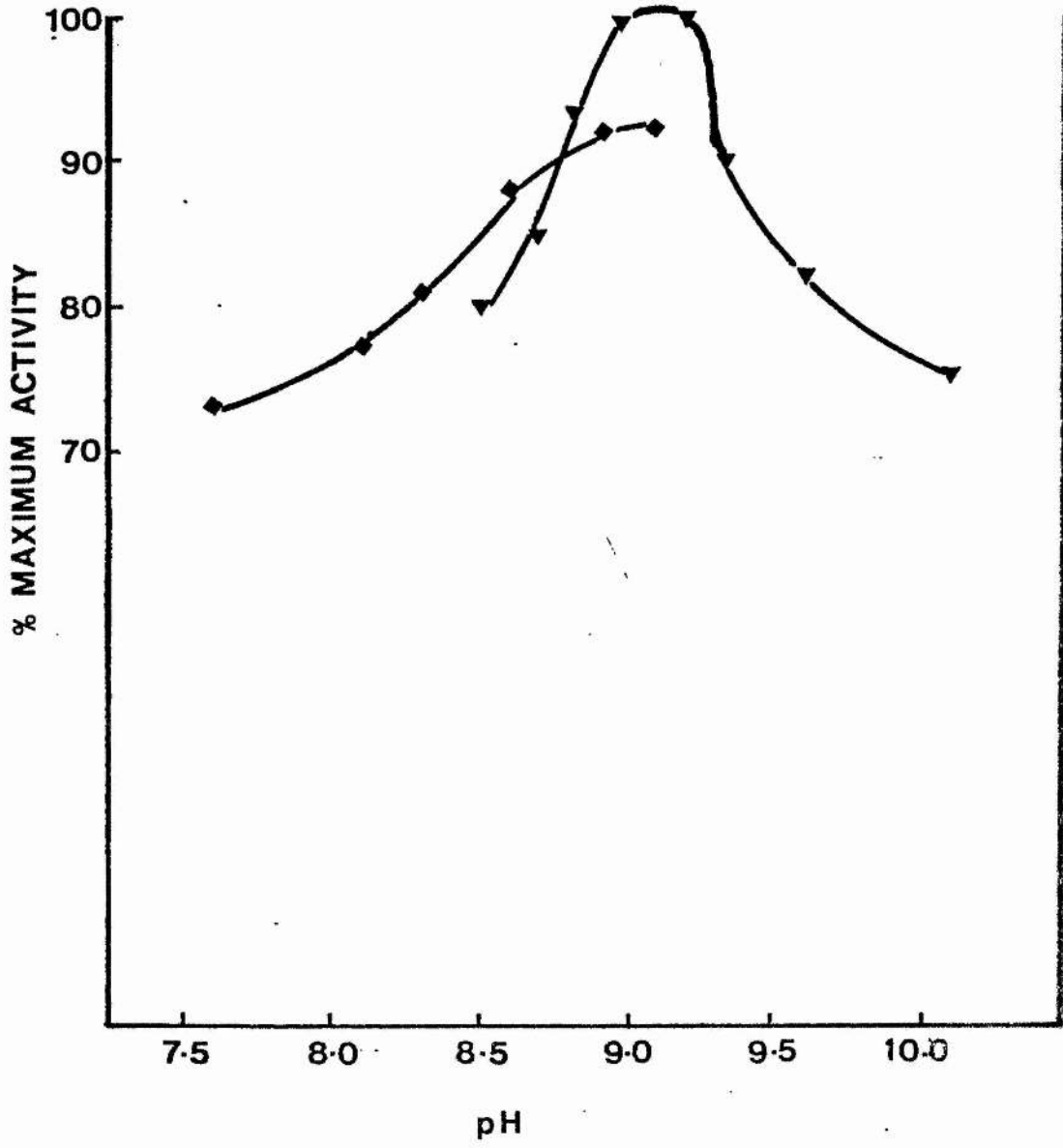


Fig. 5.2.5.6.1 pH profile of immobilised uricase. The range of pH was studied in TRIS-HCl (◆) and borate (▼) buffers.

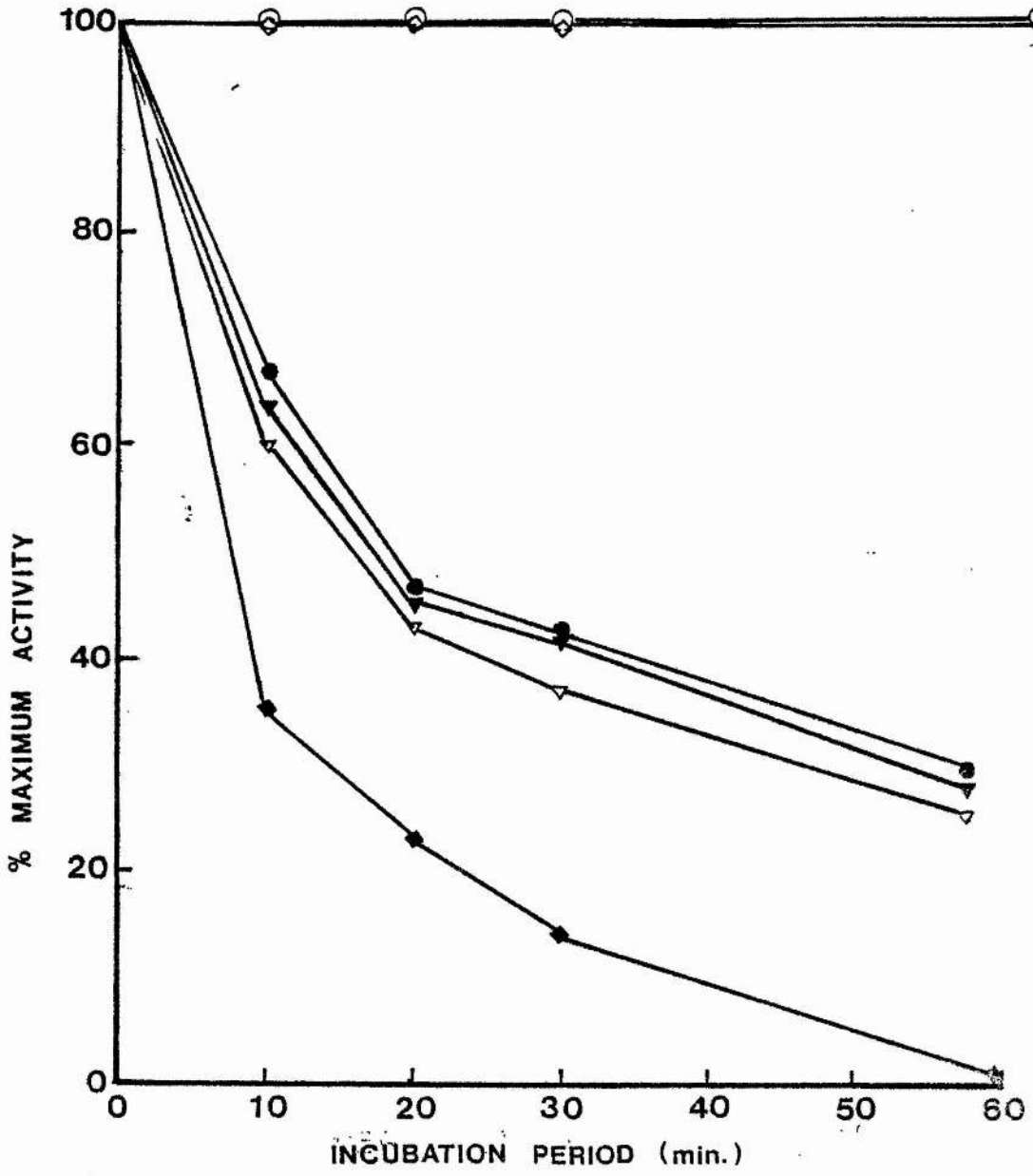


Fig. 5.2.5.7.1 Stability of immobilised uricase tube at 37° in 0.1M glycine pH 9.0 (○), 50mM glycine, 0.2M NaCl, 0.05% Triton X100 pH 9.0 (◇), 0.1M borate pH 9.0 (●), 0.1M borate pH 10.0 (◆), 0.1M borate, 0.2M NaCl pH 9.0 (▼) and 0.1M borate, 0.5M NaCl pH 9.0 (▽).

Fig. 5.2.5.7.2 shows the residual activity of the enzyme derivatives incubated in 50mM glycine, 0.2M NaCl and 0.05%(w/v) Triton X100 pH 10 under different incubation temperatures.

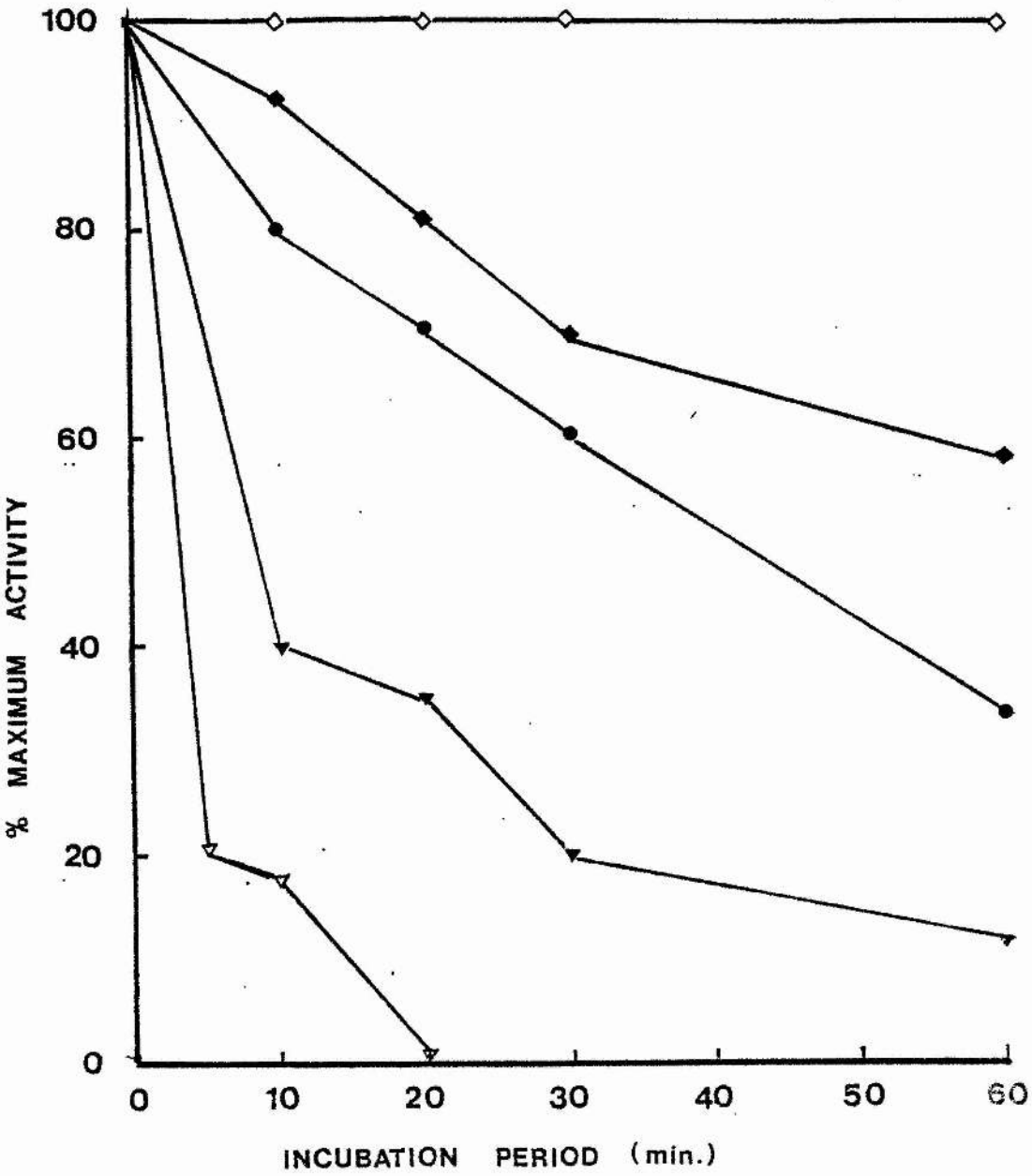


Fig. 5.2.5.7.2 Stability of immobilised uricase tube incubated in 50mM glycine, 0.2M NaCl, 0.05% Triton X100 pH 9.0 at 37° (◇), 45° (◆), 50° (●), 55° (▼) and 60° (▽).

5.3 Discussion

The method of direct activation of nylon tube by glutaraldehyde described in this work allows the generation of reactive centres on the nylon tube for enzyme immobilisation, using an easily available reagent. The conditions for activation are fairly mild and controllable.

The results suggest that the number of reactive centres increases with both time and temperature of exposure to glutaraldehyde solutions. Higher concentration of glutaraldehyde also generated more reactive centres, but the use of undiluted commercial preparations of glutaraldehyde, (25%,w/v), produced unsuitable tubes for enzyme immobilisation (when the ultimate use of these enzyme tubes were considered). Possibly the commercial preparation is unbuffered and the low pH contributes to an uncontrolled reaction on the nylon surface. Similar occurrence was observed when nylon tubes were activated by low pH glutaraldehyde solutions. This deterioration in the nylon tube may be due to simultaneous activation of nylon polymer by acid hydrolysis (87).

The chemistry of glutaraldehyde activation is very uncertain. It is notable that in this work glutaraldehyde was utilised twice, firstly to activate the nylon tube, and secondly to couple the enzyme to amine- or hydrazide-substituted nylon tube, with the conditions of reactions not exactly similar. High temperature was a feature in the activation procedure whereas the coupling

reactions were carried out at low temperatures. Onyezili (128) showed that enzyme immobilisation was in fact independent of temperature during the glutaraldehyde-mediated coupling. Whipple and Ruta (96) showed that the composition of an aqueous solution of glutaraldehyde was dependent on the temperature. Apparently the percentage of free aldehyde rose from 4% at 23° to about 70% at 70°. Presumably at 90° there is a high percentage of free aldehyde that can be involved in the activation reaction. However, the presence of polyglutaraldehyde in the alkaline solution cannot be discounted (98), though its variation with temperature is unknown. Under the circumstances, no conclusion can be made on the mechanism of glutaraldehyde reaction but the versatility of this bifunctional agent is clearly supported thus explaining its increasing use in this field.

Significantly higher activity enzyme tubes were produced using glutaraldehyde-activated nylon tubes as compared to those using TOTFB-activated nylon tubes. This trend was observed with either immobilised glucose oxidase or uricase. Apparently glutaraldehyde produced more reactive centres for spacers attachment, and eventually enzyme immobilisation. Obviously, this conclusion could not be made solely on the basis of activity retention. However, protein coupling data seemed to substantiate this point. No significant difference was observed when enzymes were immobilised by glutaraldehyde or bisimidate coupling, judging by the percentage of activity retention. Glutaraldehyde can react with a wider variety of chemical groups and may effect more enzyme coupling, but the randomness of the reactive groups used in the

coupling may deactivate some of the immobilised enzyme molecules. Imidate essentially reacts with the free amino groups in the protein molecules and may cause less denaturation. However the effectiveness of a particular method of activation or coupling may not necessarily depend on the number of reactive centres generated or the number of enzyme molecules coupled, but rather on the final activity retained on immobilisation. This factor may be finally influenced by the suitability of the particular environment created for the particular enzyme molecule.

The use of PEI as a spacer between the nylon support and the enzyme molecules may increase the number of potentially reactive groups for each reactive centre generated by glutaraldehyde activation of nylon tube. PEI is a highly branched polymer of large molecular weight (> 20,000). Its repeating unit is believed to be of the type;

$$- N - CH_2 - CH_2(NH - CH_2 - CH_2)_n - NH_2$$

The structure may be sufficiently compact to form a protective coat along the surface of the nylon tube, with free amino groups for enzyme immobilisation. Campbell et al. (90) showed that glucose oxidase was more active when immobilised to nylon tube with diaminohexane and lysine spacers than that with PEI spacer, a fact attributed to the lability of PEI in alkaline solution. It was shown here that coupling of PEI in water proved to be more useful.

There was about 20% retention of activity on immobilisation of uricase, and the tendency would be a decrease in percentage of activity retention with increasing concentration of enzyme in the coupling solution, a fact observed by Gray et al. (131) when immobilising β-glucosidase onto cellulose. Onyezili (128) showed that higher concentration of invertase resulted in a decrease in specific activity of enzyme coupled to nylon tube. No increase in total activity was achieved after a certain protein concentration. The amount of protein immobilised may be limited by the number of reactive centres available for coupling, but the total activity retention may be influenced by crowding effect of enzyme molecules. Hinberg and Driscoll (132) obtained 47% activity retention but the method of gel entrapment used is generally less destructive to enzyme molecules. Similarly, Suzuki et al. (133) obtained 43% retention of activity when uricase was electrochemically entrapped in collagen membrane. However, chemical coupling of the enzyme resulted in a lower activity retention as shown by Broun et al. (134) where 5-30% of uricase activity was retained when the enzyme was immobilised to protein membranes or porous particles by glutaraldehyde crosslinking. Gray et al. (131) achieved 18% retention of uricase activity when uricase was immobilised to cellulose by diazo coupling.

Paillot et al. (135) only achieved a 2% retention of uricase activity when the enzyme was coupled to inert protein by glutaraldehyde, but this activity retained increased to 12%

when uric acid was added to the coupling mixture; a result which the present work substantiated. Obviously uric acid and oxonate protect the active sites of the enzyme during coupling. Incidentally, Paillot et al. also showed that the protective property of urate was further enhanced when the coupling mixture was deprived of oxygen, the co-substrate of uricase.

It is interesting to note that the percentage of activity retention was similar for glucose oxidase and uricase on immobilisation to nylon tube. Though the enzymes appear to catalyse similar reactions, the enzyme molecules are dissimilar in size and conformation (?). However, there is the possibility that both enzymes are similarly affected by the nylon polymer and method of immobilisation.

The validity of the integrated Michaelis-Menten equation,

$$FS_o = K_m' \ln(1 - F) + k_t' L Q^{-1}$$

was investigated in the assay of immobilised uricase. The plots of FS_o against $\ln(1 - F)$ were linear within the range of substrate used. In order that the equation is to be applicable under the assay conditions studied, K_m' and k_t' would have to be independent of the flow rate Q, and the initial substrate concentration, S_o. Though there was no discernible effect of S_o in these experiments, there were some small variations in k_t' with ionic strength and flow rate. The variation due to ionic strength showed no particular trend with data available here.

However, there was an apparent decrease in k_t' with decreased flow rate.

The variation in K_m' was less distinct, in fact almost negligible. The K_m' obtained was about 4 times that of the soluble enzyme. Hinberg and Driscoll (132) obtained a K_m' for gel entrapped uricase which was about 3 times that of the soluble uricase, but Keys and Semersky (136) showed similar values of K_m for soluble uricase and uricase immobilised to agarose beads.

The use of short enzyme tubes may have reduced the diffusion effect, which is more prominent in packed bed reactors (137). The presence of air segmentation may also reduce the diffusion effect, but on the other hand may also affect the values of K_m' and k_t' as it may determine the dissolved concentration of oxygen in the assay solution. Hornby et al. (107) showed that the K_m and V_{max} of glucose oxidase immobilised to polystyrene tubes increased 2 fold if the enzyme tubes were assayed with oxygen-segmented substrate flow instead of air-segmented substrate flow.

It is noticeable that in Fig. 5.2.5.5.4, the plot of percentage O_2 consumed against flow rate is generally in the trend expected for the Michaelis-Menton equation. Theoretically the fraction of oxygen consumed would be equal to 1 when Q is zero, and would asymptote when Q tends to ∞.

Uricase immobilised to nylon tube has a pH optimum of 9.0-9.2. There seemed to be a slight alkaline shift on the pH profile of the immobilised uricase when compared to the soluble enzyme profile. Hinberg and Driscoll (132) found a similar but more pronounced shift in pH optimum (from 8.6 to 9.6), however uricase entrapped in collagen membrane showed an identical pH profile to the soluble enzyme (133). This difference in pH optimum may be attributed to the absence or presence of charges on the support materials.

Uricase immobilised to nylon tube was less stable to heat than its soluble counterpart when incubated in borate buffer, a fact also pointed out by McCarthy and Johnson (138) when studying uricase immobilised to elastin. Immobilised uricase incubated in glycine buffer showed more resistance to heat, comparable with the soluble enzyme. Uricase immobilised to elastin also showed comparable resistance to heat when incubated in TRIS-HCl buffer (138). Apparently TRIS and glycine have some protective properties with respect to the immobilised uricase molecules. It was also found that incubation at higher pH in borate buffer further enhanced the denaturation of the immobilised enzyme. Paillot et al. (135) achieved a better stability when uricase was crosslinked to albumin, showing that albumin could give the enzyme molecules some kind of protection.

The immobilised enzyme was found to be fairly stable at 37° when incubated in glycine buffer. The presence of NaCl and Triton X100 in this buffer, showed no observable effect on the

stability. This is significant in view of its use in the analysis of urate (section 6).

6. DEVELOPMENT OF A SYSTEM FOR THE USE OF IMMOBILISED URICASE IN AUTOMATED ANALYSIS

Uricase immobilised to nylon tube was incorporated into a continuous flow analyser (Technicon AutoAnalyser AA1). Hydrogen peroxide produced in the uricase catalysed reaction (equation 1) was reacted with two oxidative reactants, 4-aminophenazone (APZ) and dichlorohydroxyl benzoyl sulphonate (HBS), in the presence of peroxidase (POD) (equation 2).

$$\text{Uric acid} + O_2 + H_2O \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2 \qquad 1$$

$$H_2O_2 + \text{APZ} + \text{HBS} \xrightarrow{\text{POD}} \text{quinone dye} + H_2O \qquad 2$$

The coloured product was monitored at 505nm, intensity of colour produced being a measure of urate concentration.

6.1 Methods

6.1.1 Optimisation of colour reagents

Reagents

1. Working buffer; 50mM glycine, pH 9.0, containing 0.2M NaCl and 0.05%(w/v) Triton X100.

2. Reagent A: HBS; varying concentrations of HBS were dissolved in 0.2M phosphate pH 7.0.

3. Reagent B: APZ-POD; varying amounts of APZ and POD were dissolved in 0.2M phosphate pH 7.0.

4. Hydrogen peroxide standard; 0-0.25mM.

The flow system used for these studies is as shown in Fig.5.2.1.1, without the inclusion of the enzyme tube.

Keys to the flow system

Pumping line	Flow rate ($ml.min^{-1}$)
1. Air	0.8
2. Buffer	2.0
3. Sample	0.23
4. Reagent A	0.23
5. Reagent B	0.23
6. Wash line, (buffer)	1.0
7. Flow cell	2.5

Sampling rate was 60 samples h^{-1} with 2:1 sample to wash ratio. All mixing coils (MC) were maintained at 37°.

6.1.2 The use of immobilised uricase in automated analysis

Reagents

1. Standard urate solutions

50 mg uric acid and 40 mg lithium carbonate were dissolved in warm distilled water (60°), cooled and made to 100 ml. Standard urate solutions, 2-20 mg.100 ml^{-1}, were made by diluting the stock solution accordingly with distilled water.

2. Buffer solution (working buffer)

50mM glycine, pH 9.0, containing 0.2M NaCl and 0.05%(v/v) Triton X100.

3. Reagent A. HBS, 10 $mg.ml^{-1}$ in 0.2M phosphate pH 7.0.

4. Reagent B. APZ, 0.6 $mg.ml^{-1}$, and POD, 0.05 $mg.ml^{-1}$ in 0.2M phosphate pH 7.0.

5. Immobilised uricase tube; as prepared in section 5.1.3.

Procedure

The flow system used for the incorporation of immobilised uricase in automated analysis is as shown in Fig. 6.1.2.1. The system comprised solely the AutoAnalyser AA1 modules (Technicon).

Samples were automatically and continuously drawn into the continuous flow system and analysed. Working buffer was drawn between samples, to wash the sampling line.

6.1.2.1 Stability of immobilised uricase

a. Operational

2m immobilised uricase tube was incorporated into the flow system (Fig. 6.1.2.1).

Samples were prepared by adding stock urate solution to bovine serum (These samples were used as human serum was not easily available). Samples in the range of 1.5-10 mg urate.

Keys to Fig. 6.1.2.1

	Pumping line	Flow rate ($ml.min^{-1}$)
1.	Air	0.8
2.	Buffer	2.0
3.	Samples	0.23
4.	Air	0.8
5.	Buffer	2.0
6.	Buffer	0.23
7.	Reagent A	0.23
8.	Reagent A	0.23
9.	Wash line	1.0
10.	Flow cell	2.5

MC - mixing coils

ET - enzyme tube

The dialyser and mixing coils were maintained at 37°.

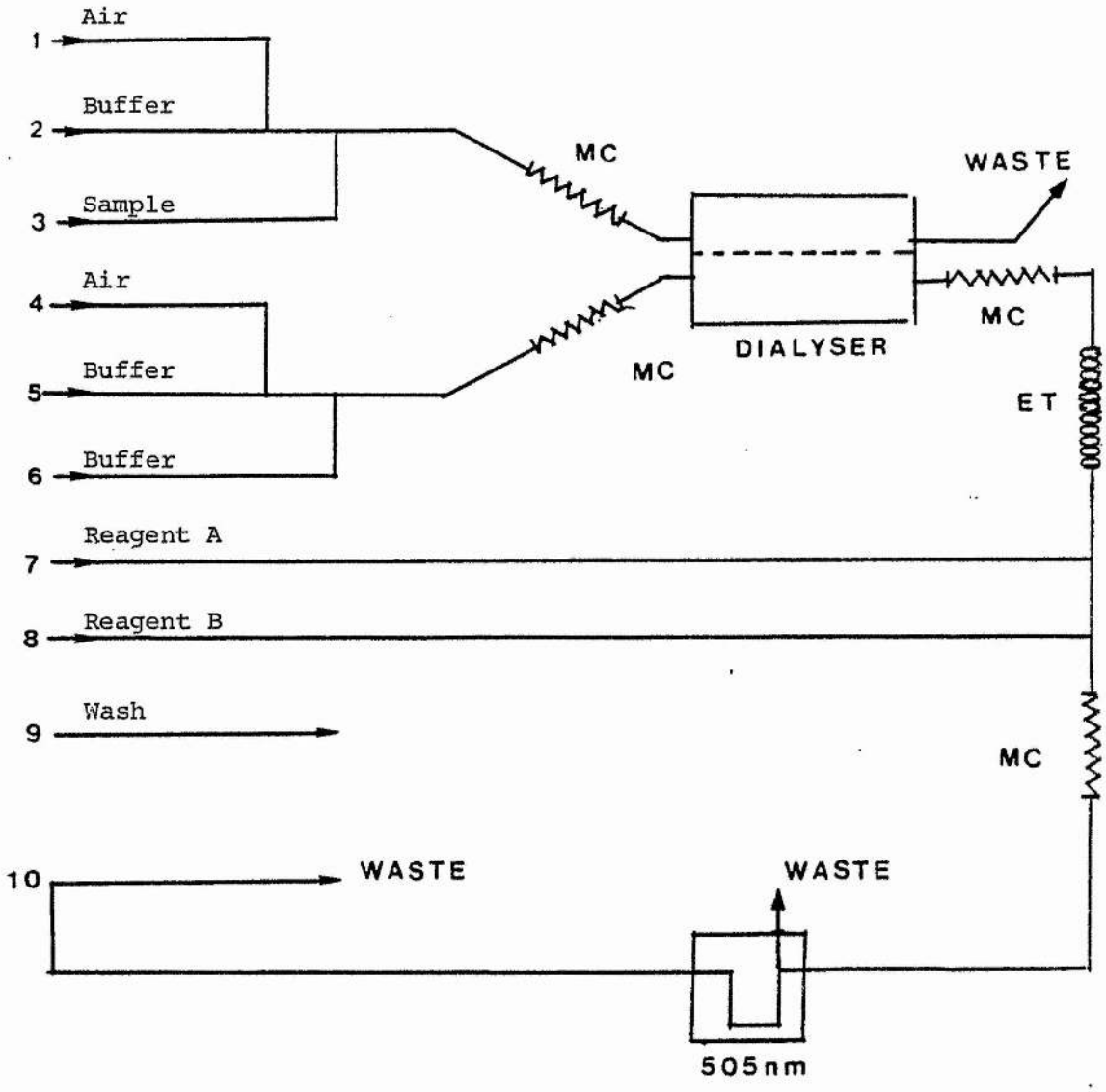


Fig. 6.1.2.1 Flow system for the incorporation of immobilised uricase tube in the automated analysis of urate.

100 ml^{-1} were assayed randomly at 60 samples h^{-1}. A total of 1500 samples were assayed continuously.

Batches of 10 to 50 samples were assayed over a period of 3 months by a single tube of immobilised uricase. The enzyme tube was stored at 4° with working buffer (when not in use). A total of 5000 samples were assayed.

b. Storage

i. Cold storage at 4°

2m uricase tubes were filled with working buffer and stored at 4°. The stability of these tubes was monitored by assaying standard solutions of uric acid.

ii. Room temperature

2m uricase tubes were filled with working buffer and kept on the bench at room temperature. The stability of the tubes were monitored by assaying standard urate solutions.

6.1.2.2 Precision

Reagents

1. Serum specimens. Samples were prepared by adding stock urate solution to bovine serum.

Procedure

25 specimens for each of three urate levels were randomly and continuously assayed on the analyser (Fig. 6.1.2.1) using 2m immobilised uricase tube. The deviations from the mean values were calculated. The coefficient of variation (C.V.) for each levels of urate gave a measure or precision.

$$C.V. = \frac{\text{Standard deviation}}{\text{mean}} \times 100$$

6.1.2.3 Recovery

Procedure

Immobilised uricase tube (2m) was incorporated into the analyser (Fig. 6.1.2.1).

Known quantities of stock urate solution were added to pooled serum. The calculated values were compared with values obtained by the immobilised enzyme method. Calibration plots were obtained using standard urate solutions with and without 7% albumin.

6.1.2.4 Carry-over studies

Carry-over studies were carried out according to the scheme put forward by Broughton et al. (139).

Reagent

1. Serum specimens; Samples were prepared by adding stock urate solution to bovine serum.

Procedure

Carry-over studies were carried out on the flow system shown in Fig. 6.1.2.1, with 2m uricase tube incorporated into the analyser.

The analysis was carried out by continuously sampling two levels of urate, proceeding with the higher level (3 specimens) followed by the lower level (3 specimens). If the specimens with the high urate level were A_1, A_2, A_3 and the specimens with the low urate levels were B_1, B_2, B_3, and the recorded values of the analyser were a_1, a_2, a_3, b_1, b_2, b_3, then the carry-over between the two samples was given as

$$K = \frac{b_1 - b_3}{a_3 - b_3}$$

where, K - carry-over constant.

b_3 was assumed as the 'true' value of specimen B since it was preceeded by 2 samples of equal concentration and thus the carry-over was negligible.

The carry-over characteristics were also studied with respect to the constituents of the working buffer. Variation in NaCl content and the concentration of Triton X100 were used to investigate their effect on sample interactions.

6.1.2.5 Interference

The presence of certain substances in serum may affect the accuracy of urate measurement. A few common substances found

in serum were studied for any interfering effect in the analysis of urate.

Procedure

Known quantities of ascorbic acid, cysteine, glutathione, albumin and glucose were added to standard urate solutions with and without 7% albumin.

The samples were assayed on the system described in Fig. 6.1.2.1, using 2m uricase tube.

6.1.2.6 Correlation

The viability of using the method in routine analysis was further investigated by comparing this method with an accepted method. Serum specimens were assayed by both methods and the data compared.

Procedure

Serum specimens were assayed by a soluble automated method on the DU PONT Automatic Clinical Analyser adapted from the method developed by Henry (25). The concentration of urate was monitored by its absorbance at 293nm. These assays were carried out at the Ninewells Hospital, Dundee.

These serum specimens were then assayed by the immobilised uricase method using the flow system shown in Fig. 6.1.2.1 with 2m immobilised uricase incorporated into the system.

As it was the practice of the Ninewells Hospital to assay a single batch of specimens weekly, the studies were carried out over a period of 4 weeks. Four batches of specimens totalling 64 specimens were assayed.

Wellcomtrol serums (Wellcome) were also assayed on the immobilised uricase system and the values obtained compared with the manufacturer's data.

6.2 Results

6.2.1 Optimum conditions for product measurement

The flow system used for these studies is as described in section 6.1.1. Absorptions at 505nm were monitored by assaying standard solutions of hydrogen peroxide and plotting a series of calibration curves.

6.2.1.1 Optimum HBS concentration

The concentration of HBS (reagent A) was varied (1 - 12 $mg.ml^{-1}$ in 0.2M phosphate buffer pH 7.0). The upper concentration studied was limited by the solubility of HBS in the buffer. The composition of reagent B was kept at 0.6 mg APZ.ml^{-1} and 0.02 mg POD.ml^{-1}. Fig. 6.2.1.1.1 shows the calibration plots obtained, with the highest absorption achieved at 12 mg HBS.ml^{-1}.

6.2.1.2 Optimum APZ concentration

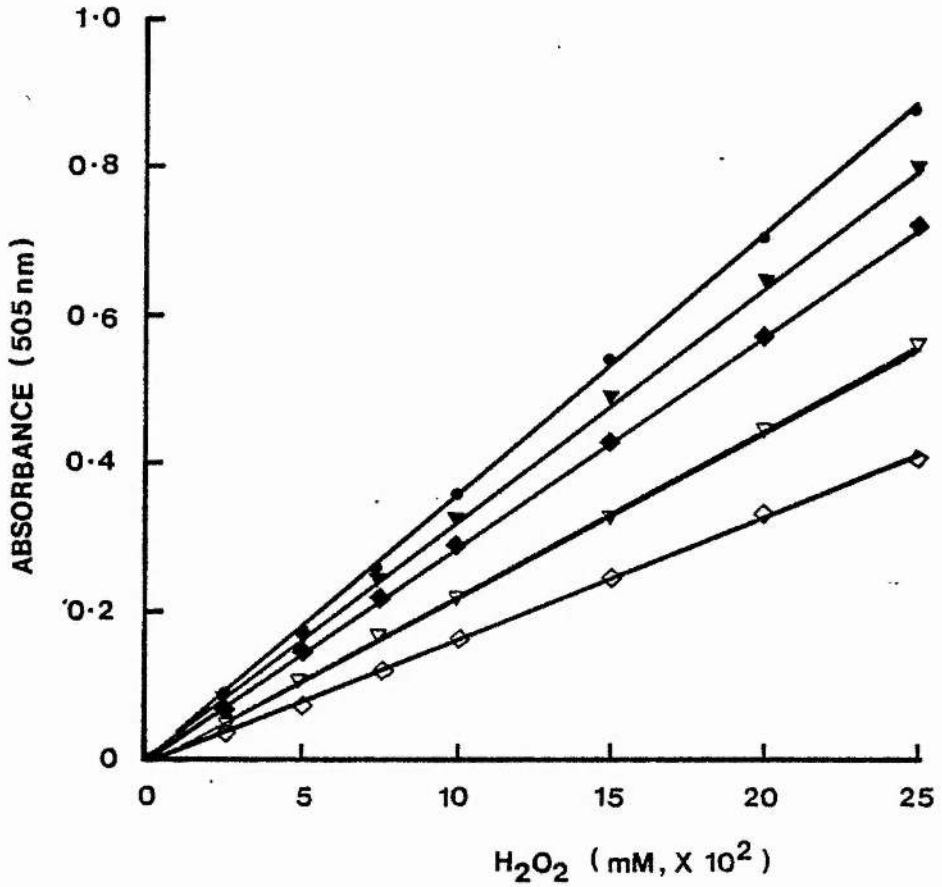
The concentration of APZ was varied (0.1 - 5.0 $mg.ml^{-1}$) with the concentration of POD kept constant (0.02 $mg.ml^{-1}$). The concentration of reagent A was maintained at 12 mg HBS.ml^{-1}. Fig. 6.2.1.2.1 shows the results obtained. Colour development was optimal at APZ concentration of 0.3 - 0.6 $mg.ml^{-1}$ with higher concentrations of APZ causing a depression in the colour development.

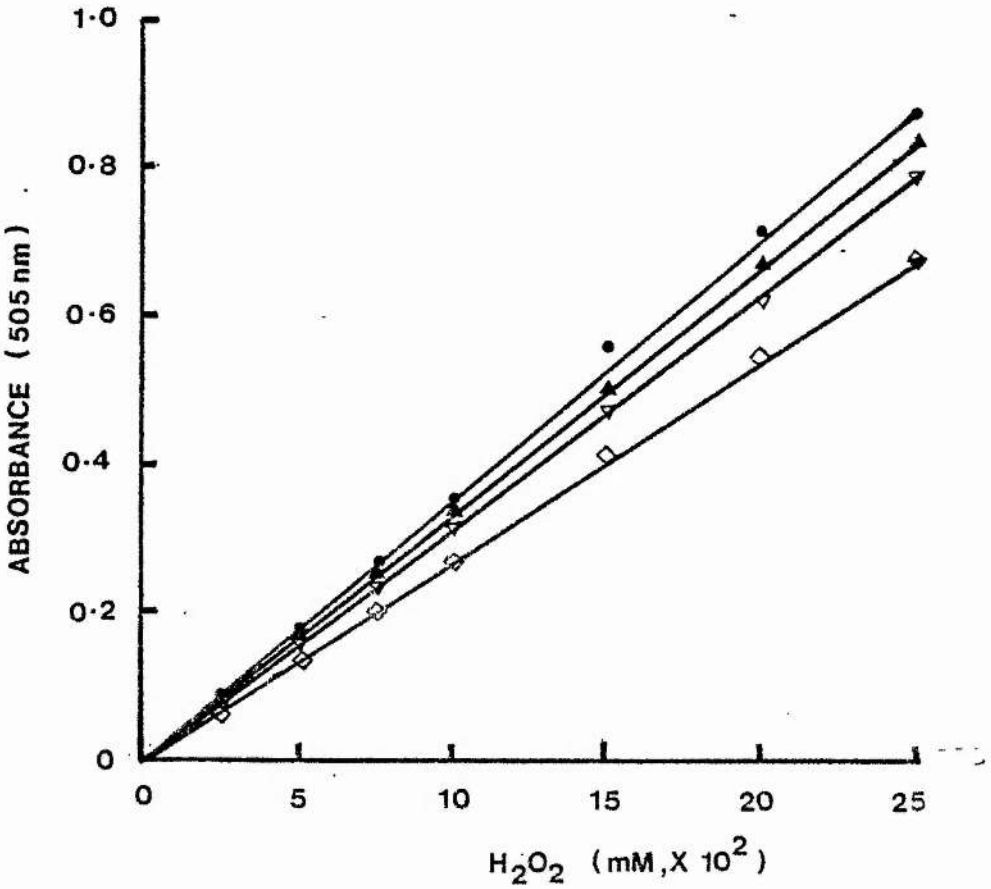
6.2.1.3 Optimum POD concentration

Fig. 6.2.1.3.1 shows the results obtained when the hydrogen peroxide standard solutions were assayed with different concentrations

Fig. 6.2.1.1.1 Calibration plots for different concentrations of HBS. The concentrations of HBS used were 1 $mg.ml^{-1}$ (◇), 2 $mg.ml^{-1}$ (▽), 6 $mg.ml^{-1}$ (◆), 10 $mg.ml^{-1}$ (▼) and 12 $mg.ml^{-1}$ (●).

Fig. 6.2.1.2.1 Calibration plots for different concentrations of APZ. Plots are for APZ concentration of 0.1 $mg.ml^{-1}$ (▽), 0.6 $mg.ml^{-1}$ (●) and 1.0 $mg.ml^{-1}$ (▲) and 5.0 $mg.ml^{-1}$ (◇).


1·0
0·8
0·6
0·4
0·2
0
ABSORBANCE (505 nm)
0
5
10
15
20
25
H_2O_2 (mM, X 10^2)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ABSORBANCE (505 nm)
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H_2O_2 (mM, X 10^2)

of POD (0.005 - 1.0 mg.ml^{-1}). APZ concentration was maintained at 0.6 mg.ml^{-1}, and the HBS concentration was kept constant at 12 mg.ml^{-1}. No increase in colour development was observed at POD concentration higher than 0.02mg.ml^{-1}.

6.2.1.4 Buffer used in the flow system

Borate buffer was considered as an alternative working buffer for use in the flow system. 50mM borate pH 9.0, containing 0.2M NaCl and 0.05%(v/v) Triton X100 was used.

Fig. 6.2.1.4.1 shows the calibration plots obtained with the two buffer systems. There was better colour development when standard hydrogen peroxide solutions were assayed in glycine buffer. Although the difference was small, it was consistently obtained.

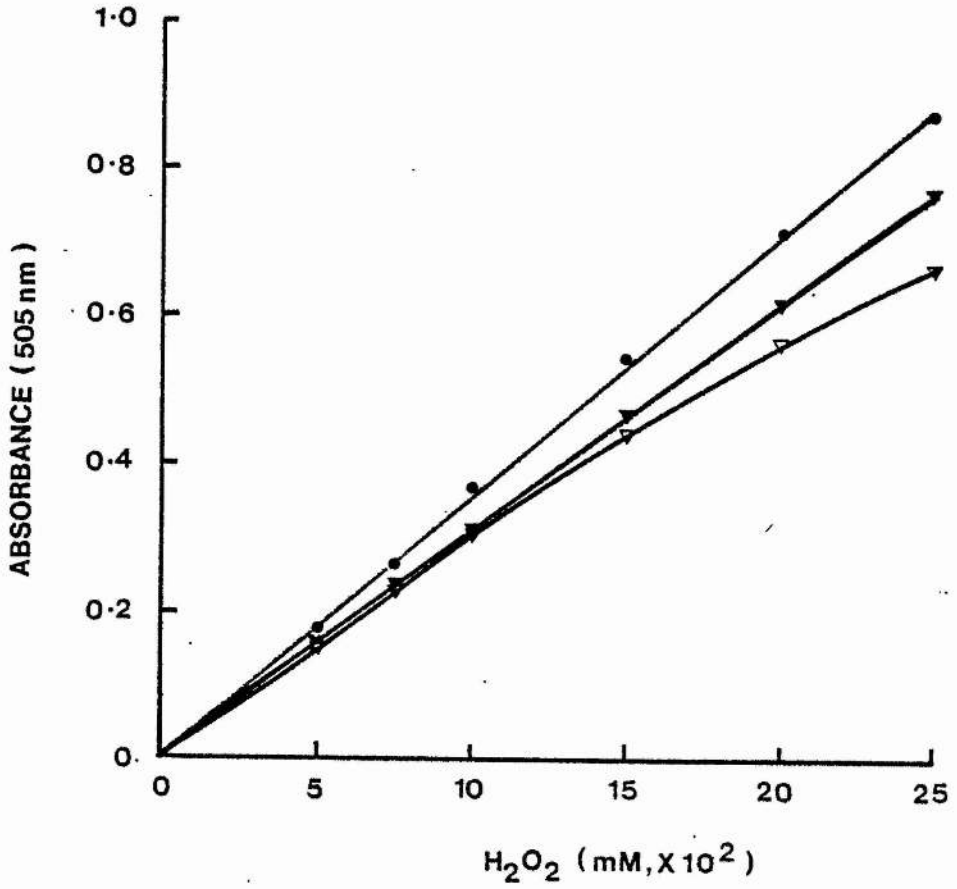
6.3.3 Comparison between the HBS-APZ reagent and Trinder's reagent

For the HBS-APZ reagent, 12 mg HBS.ml^{-1}, 0.6 mg APZ.ml^{-1} and 0.02mg POD.ml^{-1} were used, while Trinder's reagent was as described in section 5.1.2.1.

The results are presented in Fig. 6.2.2.1 which clearly shows the increased sensitivity of the HBS-APZ reagent (about 3 fold).

Fig. 6.2.1.3.1 Calibration plots for different concentrations of POD. The plots are for POD concentration of 0.005 $mg.ml^{-1}$ (▽), 0.01 $mg.ml^{-1}$ (▼) and 0.02 $mg.ml^{-1}$ (●).

Fig. 6.2.1.4.1 Calibration plots using two different buffer systems. The buffers used were borate (◆) and glycine (●).


1·0
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0·6
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ABSORBANCE (505 nm)
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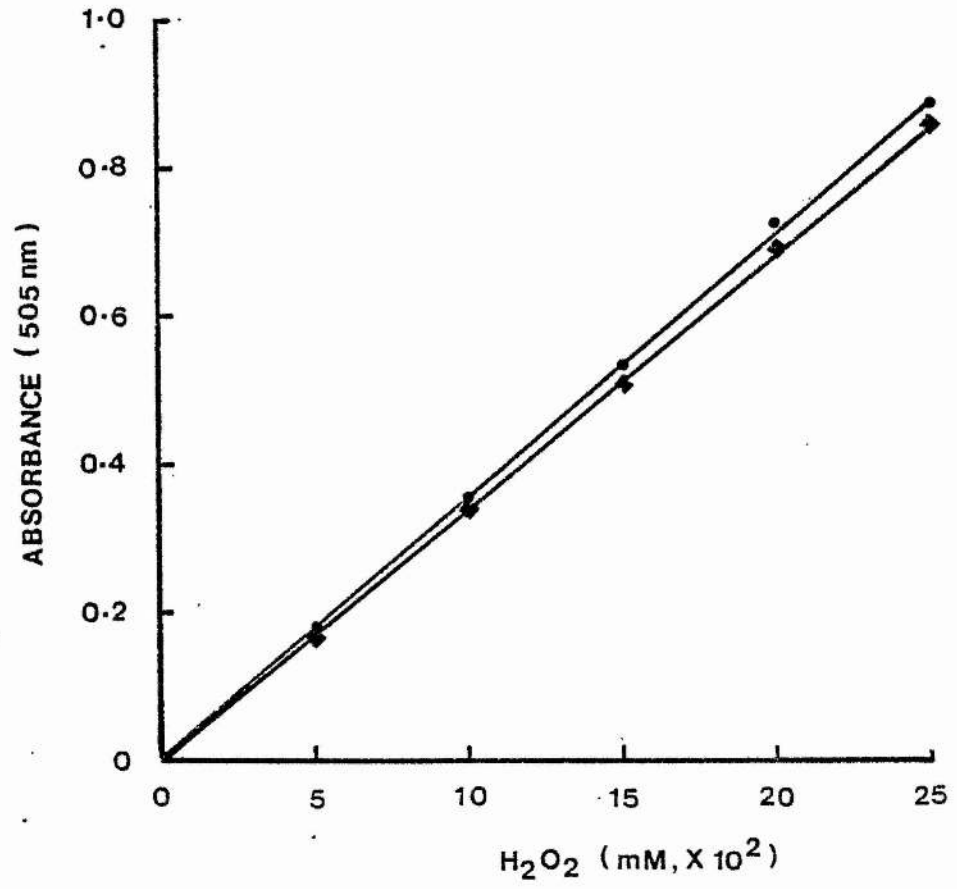
25
H_2O_2 (mM, X 10^2)


1·0
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ABSORBANCE (505 nm)
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H_2O_2 (mM, X 10^2)

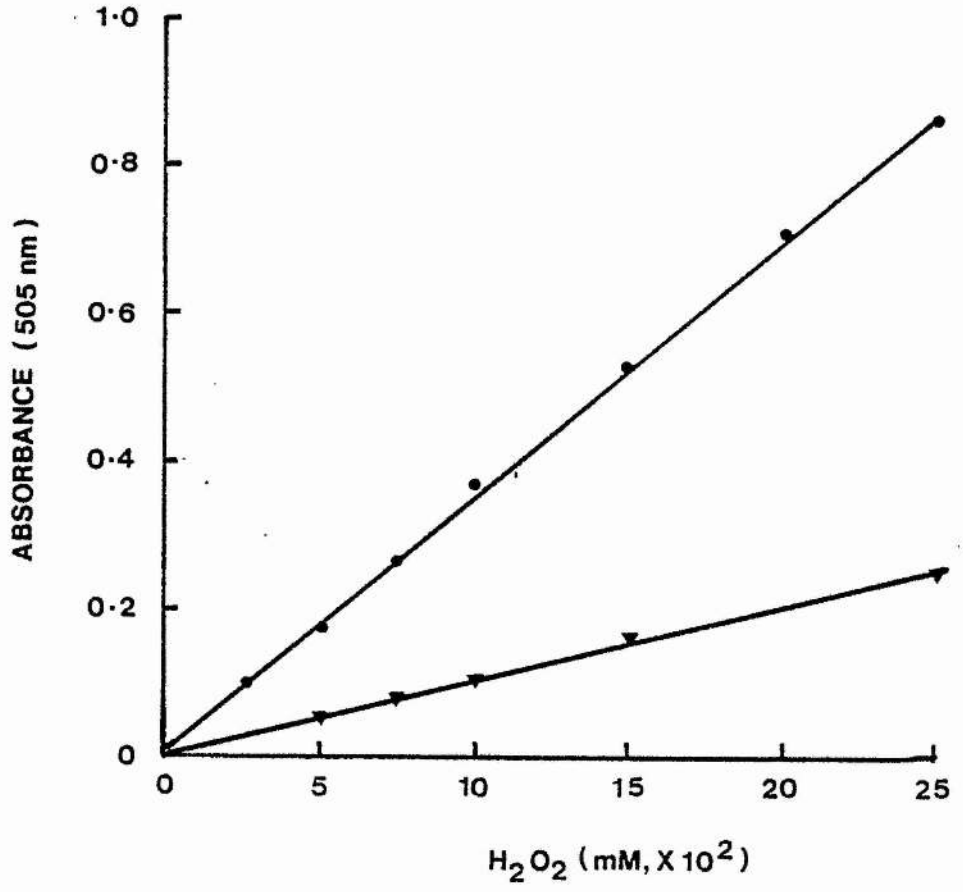

Fig. 6.2.2.1 Calibration plots obtained with different chromogens for the colour-forming reactions. HBS-APZ reagent (•) and Trinder's reagent (▼) were used for comparison of sensitivity.

6.2.3 Use of immobilised uricase in analysis

6.2.3.1 Calibrations of analytical system

Immobilised uricase tubes were incorporated into the flow system shown in Fig. 6.1.2.1 and standard solutions of urate assayed as described in section 6.1.2. Fig. 6.2.3.1.1 shows a typical calibration plot of standard solutions of urate, when 2m immobilised uricase tube was incorporated into the analyser. This kind of calibration plot was used for determination of urate levels in serum.

Fig. 6.2.3.1.2 shows calibration plots obtained when uricase tube with different activity (k_t) were incorporated into the analyser.

Fig. 6.2.3.1.3 shows a series of plots obtained when 0.5, 0.1 and 2m of a particular uricase tube were used to assay standard urate solutions.

Fig. 6.2.3.1.4a, b shows typical recorder outputs on assaying standard urate solutions.

6.2.3.2 Stability of immobilised uricase

a. Operational

Operational stability of uricase was monitored as described in section 6.1.2.1a. After assaying 1500 serum samples continuously, no change in the calibration plot was observed.

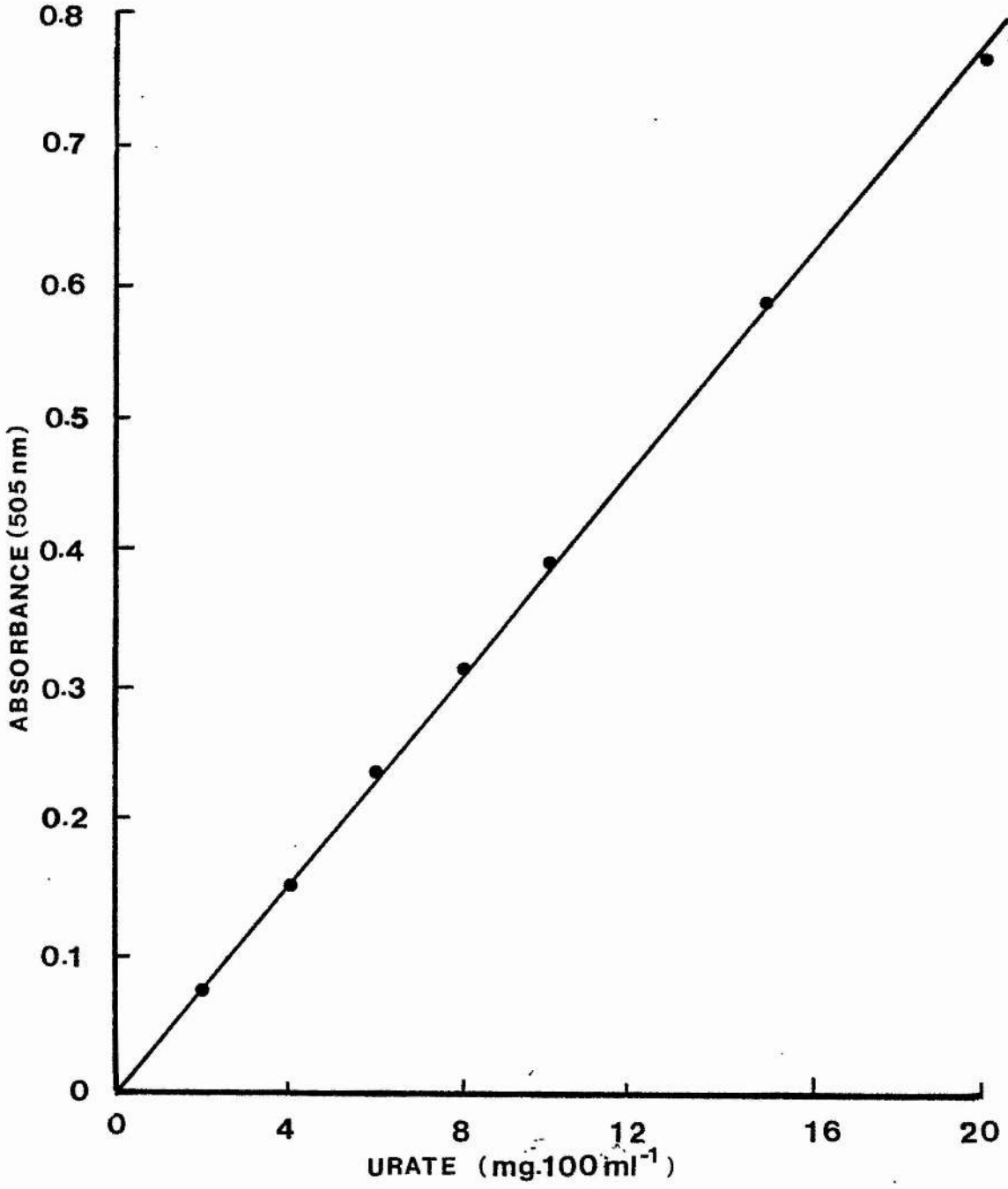

Fig. 6.2.3.1.1 Calibration plot for determination of urate levels in serum. Standard solutions of urate were assayed at the rate of 40 samples.h^{-1}.

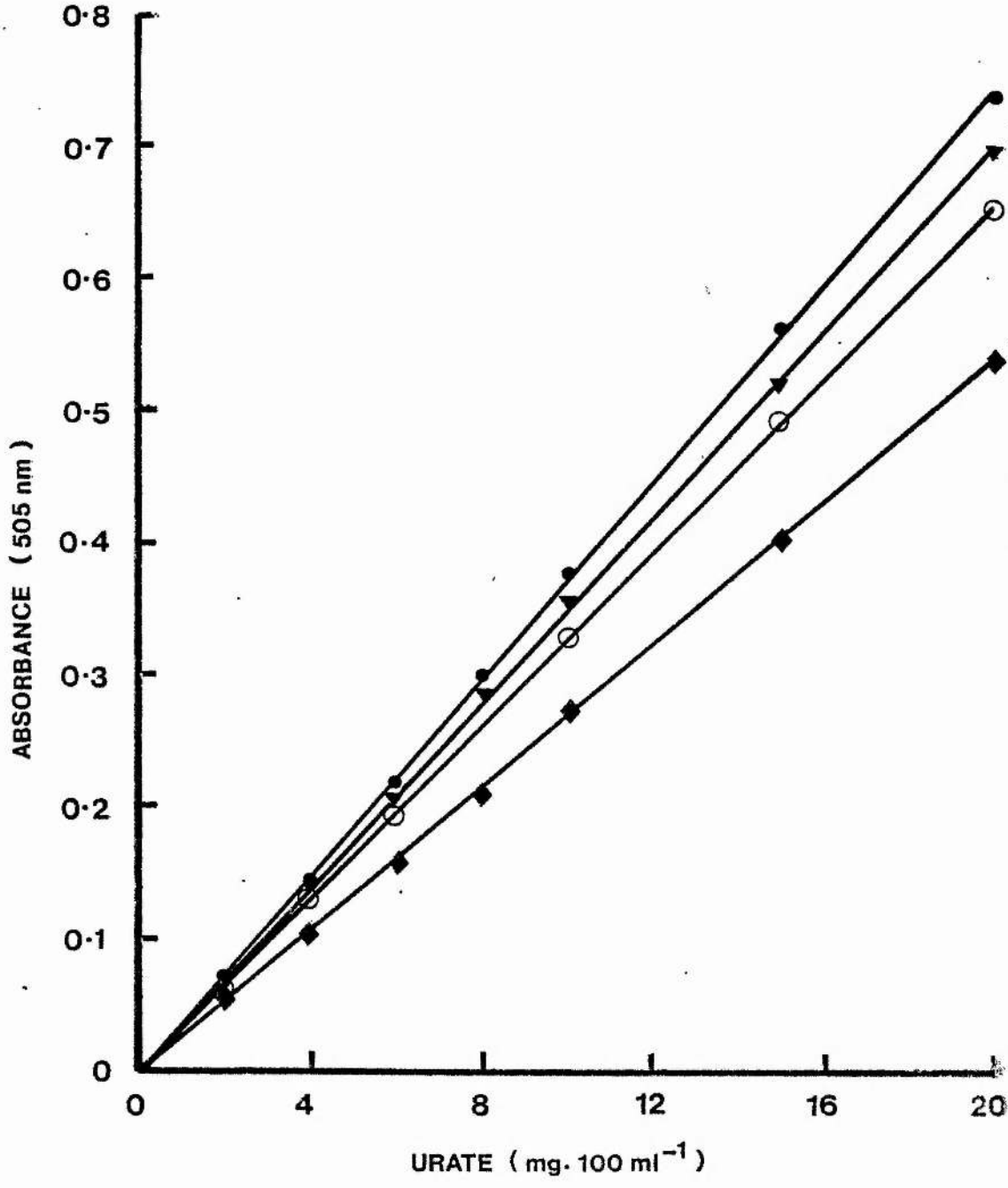

Fig. 6.2.3.1.2 Calibration plots obtained by using uricase tubes of different activities. k_t values of the enzyme tubes were 0.39 U.m^{-1} (●), 0.35 U.m^{-1} (▼), 0.23 U.ml^{-1} (○), and 0.11 U.m^{-1} (◇). For further details of the different tubes used, see Table 5.2.5.3.1.

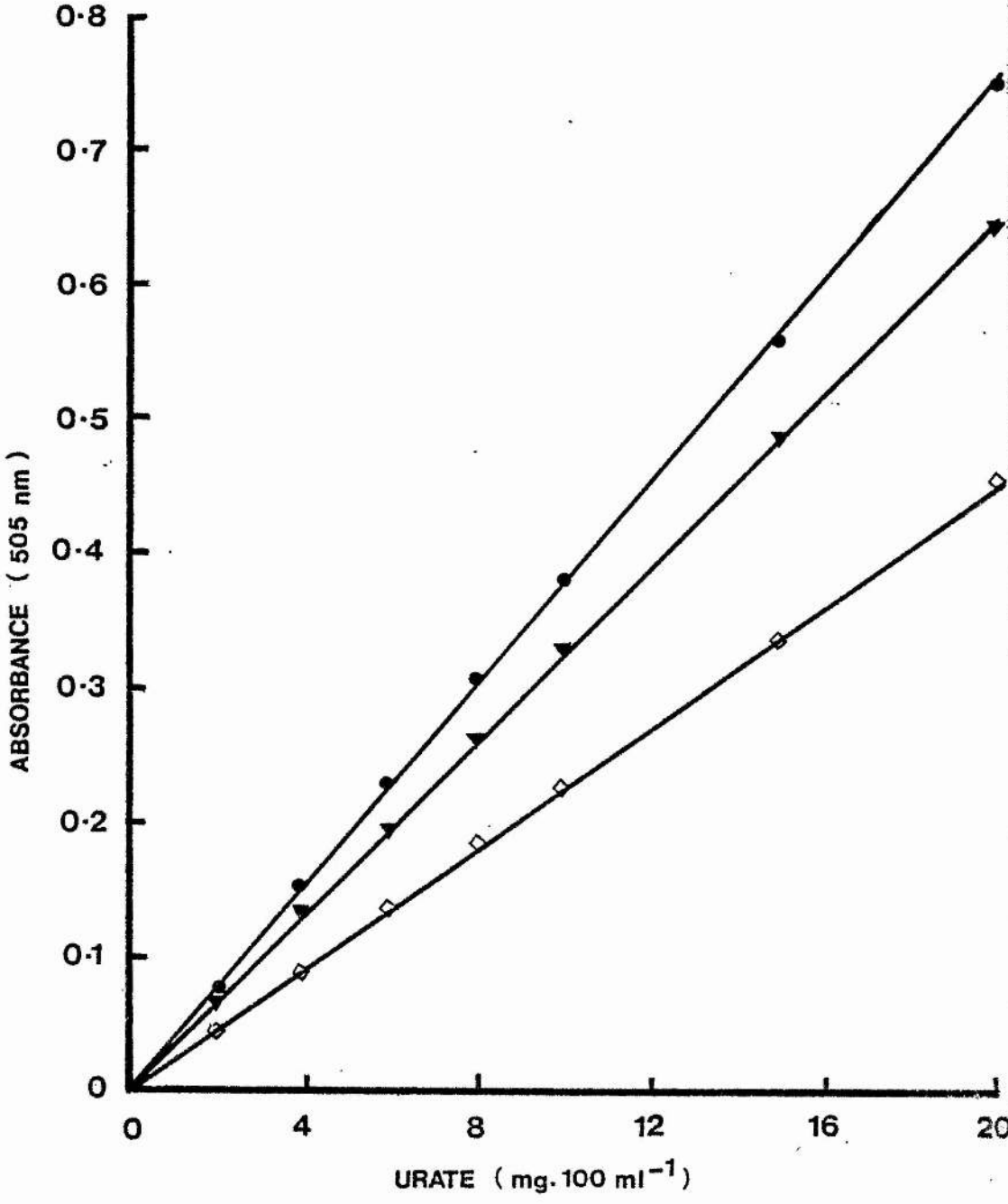

Fig. 6.2.3.1.3 Calibration plots obtained by using different lengths of an immobilised enzyme tube. 0.5m (◇), 1.0m (▼), and 2.0m (●) lengths of the uricase tube were used.

Fig. 6.2.3.1.4a Data presentation for automated analysis of urate using immobilised uricase. The above figure shows the recorder output when standard solutions were assayed, at 40 samples $h.^{-1}$ with 2:1 sample to wash ratio.

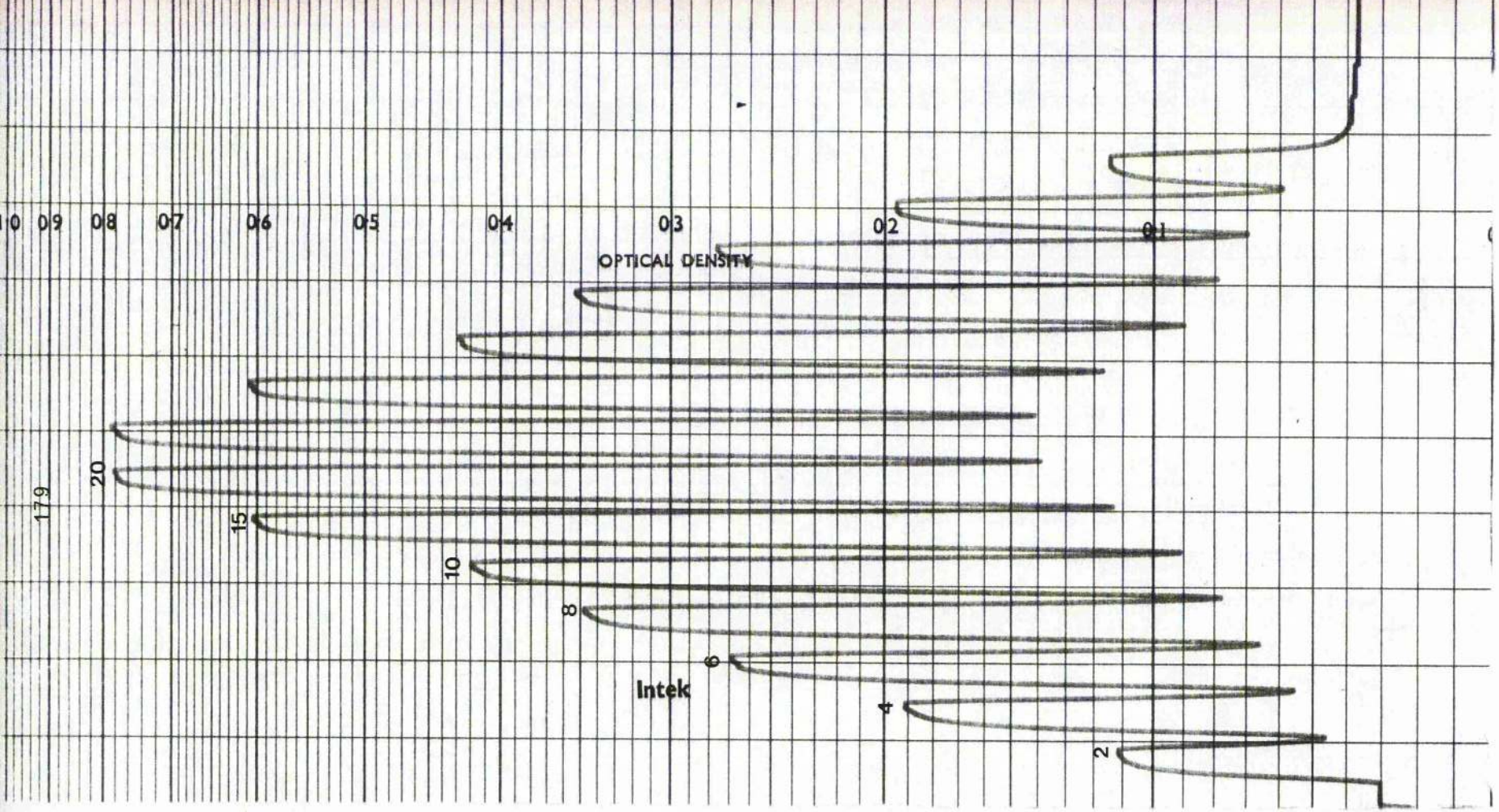
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Fig. 6.2.3.1.4b Data presentation for automated analysis of urate using immobilised uricase. The figure shows the recorder output when standard solutions of urate were assayed, at 60 samples h^{-1} with 2:1 sample to wash ratio..

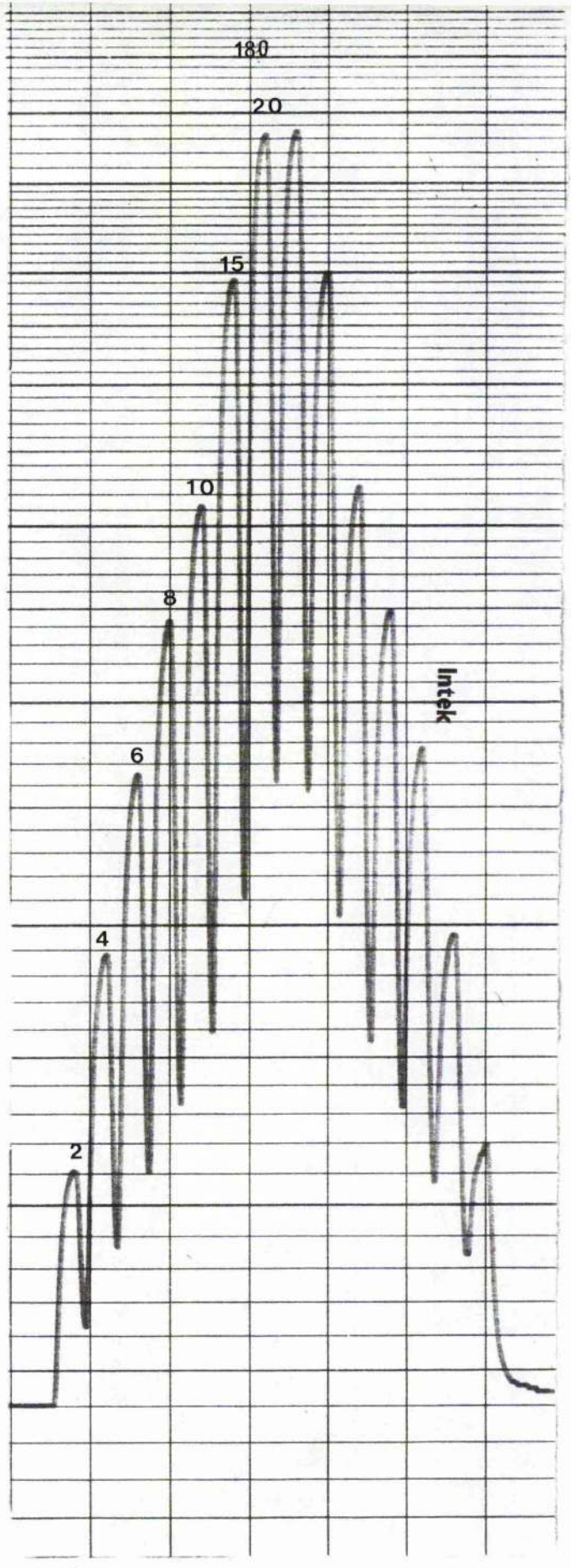
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Fig. 6.2.3.2.1 shows the changes in the calibration plots over a period of 90 days with intermittent use of the immobilised uricase tube, during which a total of 5000 serum samples were assayed.

b. Storage

i. Cold storage at 4°

Fig. 6.2.3.2.2 shows the calibration plots obtained with enzyme tubes stored at 4° over a period of 90 days. Although there was apparent decrease in activity the calibration plots were linear within this period.

ii. At room temperature

Fig. 6.2.3.2.3 shows the calibration plots obtained with enzyme tubes exposed to room temperature. The plots seemed to be linear up to 14 days, after which the activity loss was sufficiently large to affect the linearity of the calibration plots.

6.2.3.3 Precision studies

The precision of this analytical system was studied and the results summarised in Table 6.2.3.3.1. Overall, the urate levels obtained showed a higher mean value when the serum specimens were assayed at the higher sampling rate. The increase in mean values may be attributed to the false increase in readings due to sample interactions (section 6.2.3.5).

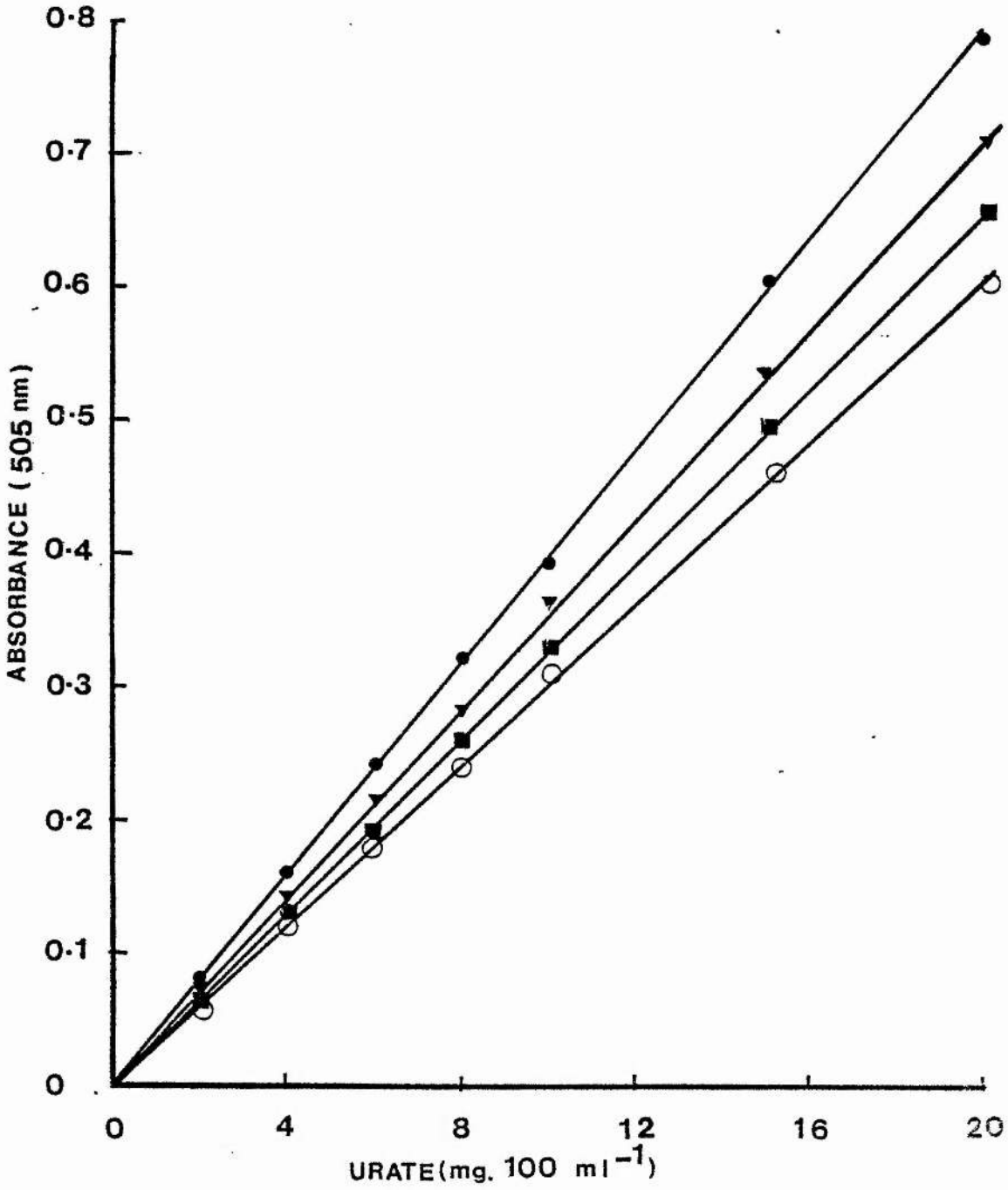

Fig. 6.2.3.2.1 Operational stability of immobilised uricase. Intermittent assay of serum specimens were carried out, and the enzyme tube stored at 4° when not in use. The calibration plots were obtained after 0-14 days (●), 45 days (▼), 60 days (◈), and 90 days (○).

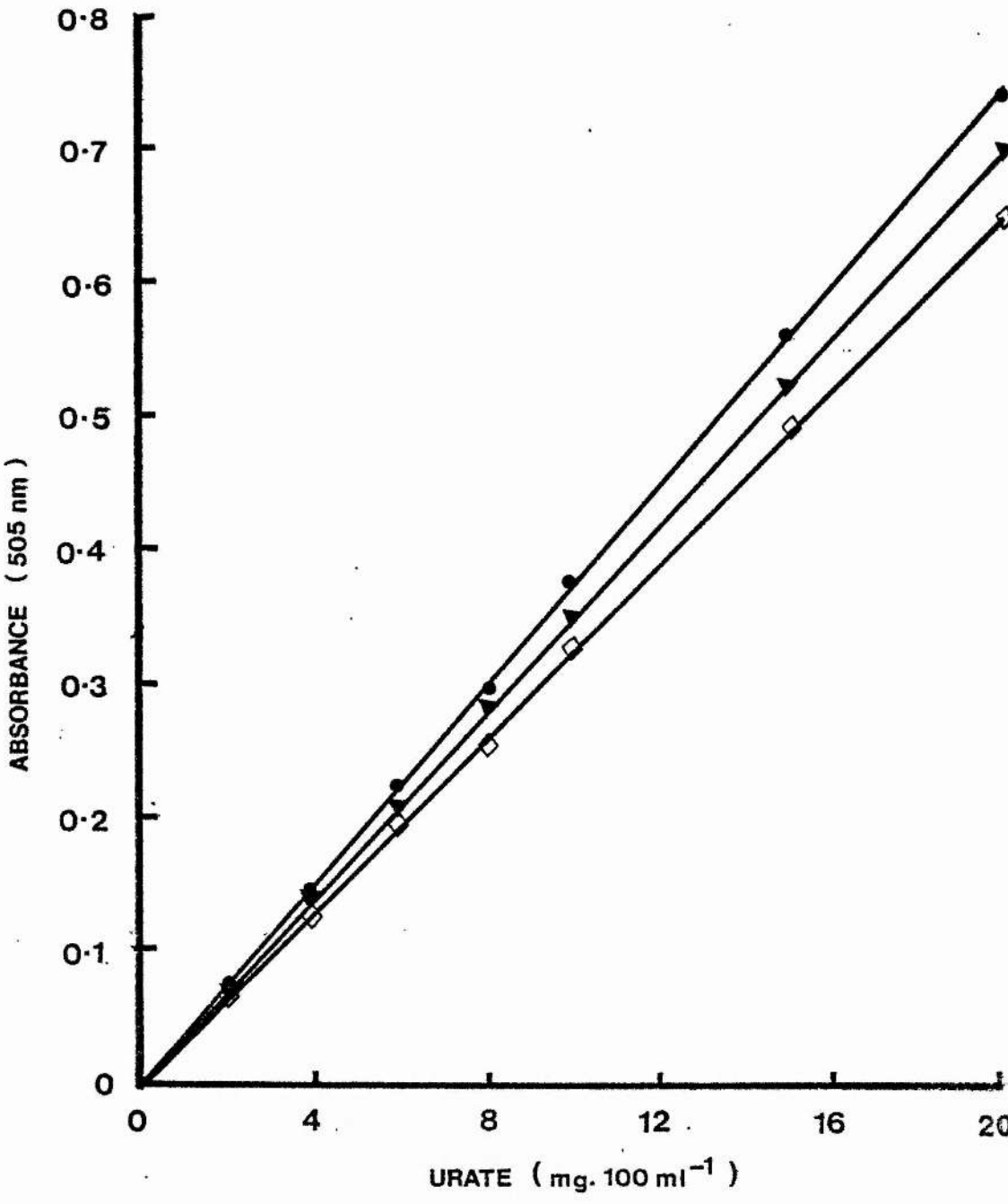

Fig. 6.2.3.2.2 Storage stability of immobilised uricase at 4°. The immobilised uricase tubes were assayed initially , and after 30 days (●), 60 days (▼) and 90 days (◇).

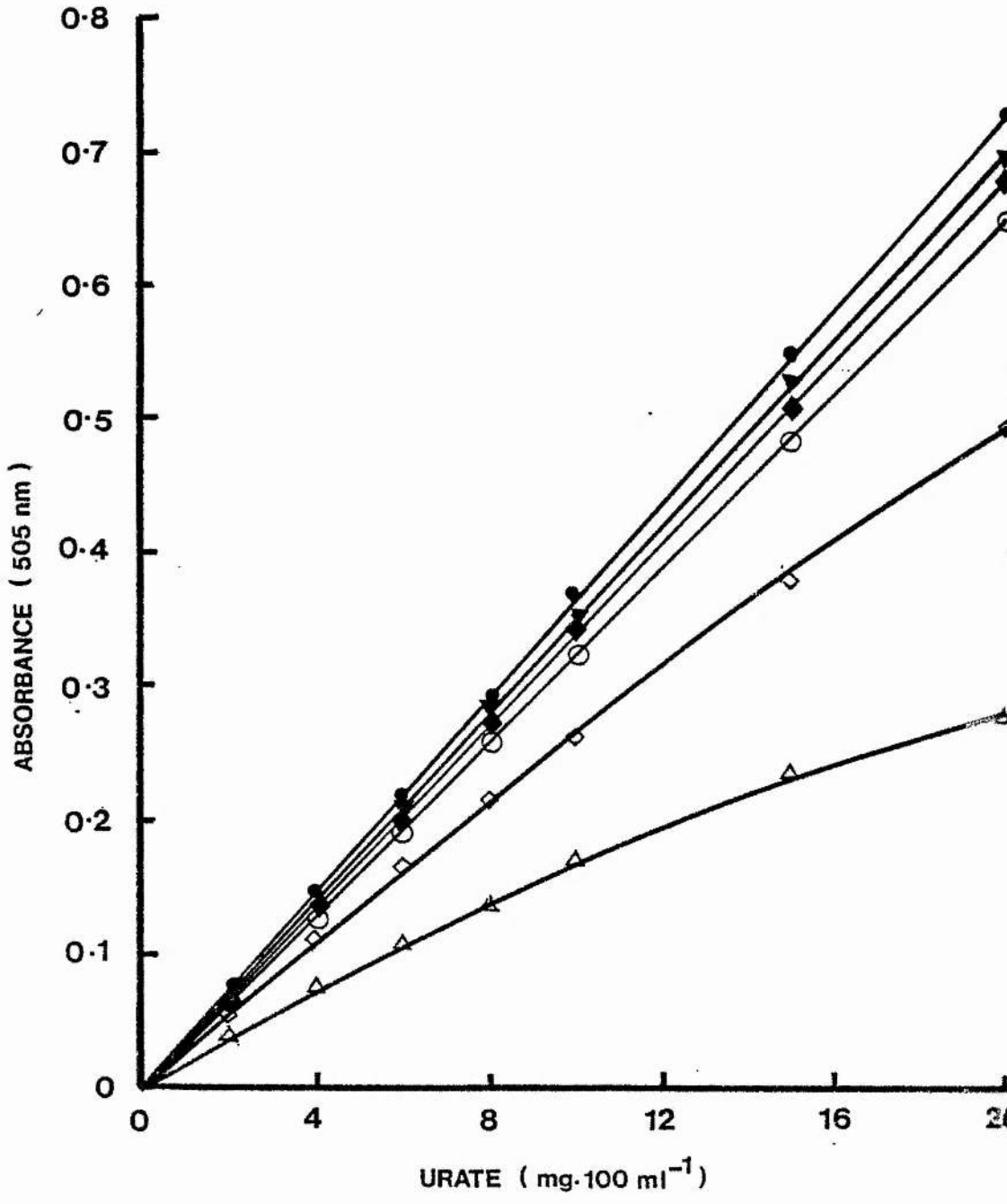

Fig. 6.2.3.2.3 Storage stability of immobilised uricase at room temperature. Uricase tubes were assayed initially (●) and after 2 days (▼), 7 days (◆), 14 days (○), 30 days (◇) and 60 days (△).

Sampling rate: 60 samples.h^{-1}

Mean	Standard deviation	Coefficient of variation
2.416	0.116	4,8
4.570	0.106	2.3
10.104	0.203	2.0

Sampling rate: 40 samples.h^{-1}

Mean	Standard deviation	Coefficient of variation
2.308	0.081	3.5
4.528	0.102	2.25
10.092	0.076	0.75

Table 6.2.3.3.1 Precision data for the analysis of urate by the immobilised uricase method. A 2:1 sample to wash ratio was used for both rates of sampling. The urate values are in mg.100ml^{-1} . For further details of this study see section 6.1.2.2.

This characteristic was further demonstrated by the higher values of standard deviations obtained with a higher sampling rate. Increase in standard deviations were sufficiently high to cause an increase in the coefficient of variations (C.V.). However, the coefficient of variations obtained at both sampling rates were still less than 5% for all three urate levels investigated.

6.2.3.4 Recovery

The recovery of urate added to samples was studied. Stock solution of urate was added to pooled serum, and the total urate assayed by the immobilised uricase system compared with total urate available (by calculation). The results were calculated by using two calibration plots, one using protein-free standard solutions of urate and another using standard urate solutions containing 7% albumin.

The results summarised in Table 6.2.3.4.1 show virtually 100% recovery of urate when the system was calibrated with protein-free standard solutions, and 99% recovery when using standard solutions with 7% albumin.

Serum samples $mg.100ml^{-1}$	Urate added $mg.100ml^{-1}$	Total		% Recovery
		calculated $mg.100ml^{-1}$	assayed $mg.100ml^{-1}$	
4.5	2.29	6.79	6.8	100.1
4.5	3.2	7.7	7.8	101.2
5.4	2.29	7.69	7.7	100.2
5.4	3.2	8.6	8.7	101.2
		Average recovery -		100.7

A: Calibration with protein-free standard solutions.

Serum samples * mg.100ml^{-}	Urate added mg.100ml^{-}	Total		% Recovery
		calculated mg.100ml^{-}	assayed mg.100ml^{-}	
4.4	2.29	6.69	6.6	98.6
4.4	3.2	7.6	7.6	100.0
5.4	2.29	7.69	7.6	98.8
5.4	3.2	8.6	8.5	98.8
		Average recovery -		99.1

B: Calibration with urate standard solutions containing 7% albumin.

Table 6.2.3.4.1 Recovery data obtained from the immobilised uricase method. (*) assayed by the immobilised uricase method. Each assayed value was the average of 4 determinations.

6.2.3.5 Carry-over studies

Carry-over characteristics of the system were investigated as described in section 6.1.2.4.

Fig. 6.2.3.5.1 illustrates a typical recorder output for these studies.

The carry-over coefficients for sampling rate of 40 and 60 h^{-1} were obtained with urate samples of 10 mg.100ml^{-1} and 2 mg.100ml^{-1}. Table 6.2.3.5.1 summarises the results obtained.

Fig. 6.2.3.5.1 Recorder output for the studies of carry-over characteristic of the analytical system. The figure shows the output for standard urate solutions, followed by 6 urate samples. See section 6.1.2.4 for details.

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b2
b3

Sampling rate (h^{-1})	Carry-over coefficient (K)
40	1.27
60	2.5

Table 6.2.3.5.1 Carry-over coefficient at different sampling rates. Each value was the average of 3 determinations.

The concentrations of NaCl and Triton X100 in the working buffer were varied and the carry-over characteristics were studied by calculating the carry-over coefficient under the conditions studied.

Table 6.2.3.5.2 summarises the results obtained.

NaCl (M)	Carry-over coefficient (K)
0.1	1.5
0.2	1.25
0.3	1.24
0.4	1.05
0.5	1.1

A: Effect of varying NaCl concentration in the working buffer. The concentration of Triton X100 was maintained at 0.05%(v/v).

Triton X100 (%,v/v)	Carry-over coefficient (K)
0.001	1.37
0.025	1.17
0.05	1.16
0.1	1.16

B: Effect of varying concentration of Triton X100 in the working buffer. 0.2M NaCl was maintained in the buffer.

Table 6.2.3.5.2 Carry-over coefficients in the presence of different concentrations of NaCl and Triton X100 in the working buffer. Each K value was the average of 3 determinations. Sampling rate: $40h^{-1}$.

From Table 6.2.3.5.2, it can be seen that in the concentration range studied, the carry-over coefficients showed little variation. It was observed however that in the total absence of NaCl, there was very high sample interaction (K varied from 30 to 50). Significantly, there was also much reduced dialysis efficiency. The amount of urate dialysed varied but generally was less than 50% of maximum efficiency. It was observed that in the presence of 0.4M and 0.5M NaCl, a slight depression in absorbance was obtained (a decrease of about 5% of maximum absorbance).

In the total absence of Triton X100 (but in the presence of 0.2M NaCl) the carry-over coefficient was still fairly low (approximately 1.5). However, there was an apparent effect on the efficiency of dialysis. The urate dialysed may be

reduced to about 70% of maximum efficiency, but this effect was not consistently observed.

6.2.3.6 Interference

The effect of certain compounds on the 'true' levels of urate in serum was studied (section 6.1.2.5) and the results summarised in Table 6.2.3.6.1.

Substance added	Concentration $mg.100ml^{-1}$	Urate (protein-free- $mg.100ml^{-1}$	%	Urate (7% albumin) $mg.100ml^{-1}$	%
-	-	5	100	-	-
L-ascorbic	2	3.8	76	4.8	96
acid	5	3.2	64	3.6	73
L-cysteine	5	4.8	96	5.0	100
L-glutath-					
ione	5	4.6	92	5.0	100
Albumin	7000	5.15	103	-	-
Glucose	300	5	100	5.05	101
-	-	10	100		
L-ascorbic	2			9.6	96
acid	5			8.3	83
Albumin		10.1	100.1		

Table 6.3.3.6.1 Interference effect of some substances in the assay of urate by the immobilised uricase method. Each value is the average of 4 determinations.

6.2.3.7 Correlation studies

The studies were carried out as described in section 6.1.2.6. The data obtained from the present method and the reference method yielded the linear regression equation,

$$y = 1.011x - 0.046$$

where the correlation coefficient, $r = 0.984$, $\bar{y} = 5.584$, $\bar{x} = 5.567$, (bias = 0.3%) for 64 serum specimens over the range of 1.5 - 10.8 mg urate.$100ml^{-1}$.

Fig.6.2.3.7.1 shows the data obtained from the two methods.

Table 6.2.3.7.1 shows the values obtained when control serum were assayed on the immobilised uricase system. The results show, in general, lower values of urate obtained by the present method.

Wellcomtrol serum	Manufacturer's data mg.$100ml^{-1}$	Immobilised uricase method mg.$100ml^{-1}$
No: 1	5.6	5.15
No: 2	8.5	8.15
No: 1 & 2 (1:1,v/v)	7.05	6.55

Table 6.2.3.7.1 Comparative data obtained with control serums.

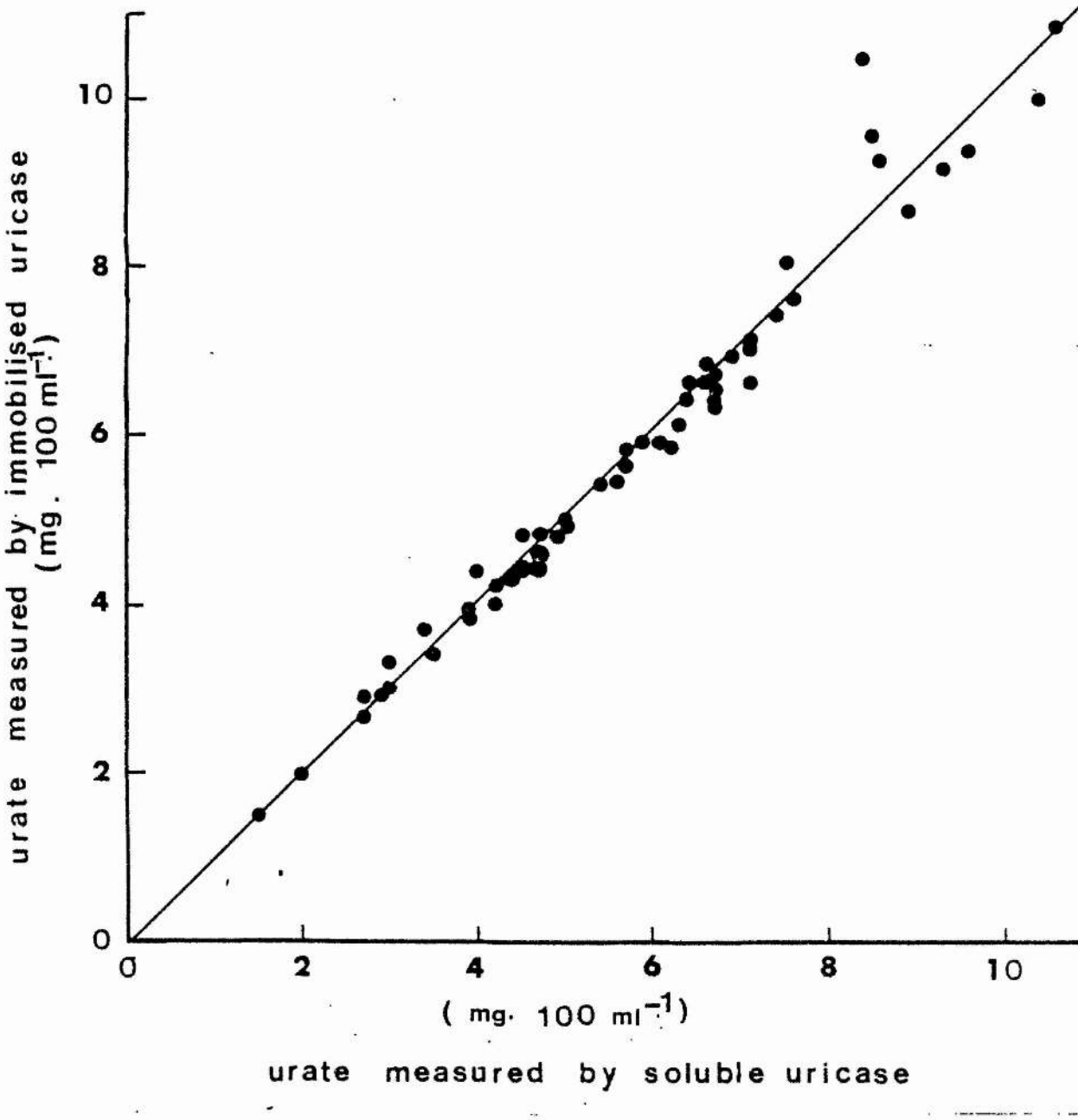

Fig. 6.2.3.7.1 Plots of data obtained by the immobilised uricase method and a soluble uricase method.

6.3 Discussion

The use of uricase immobilised to nylon tube in the automated analysis of urate in serum was demonstrated.

Linear calibration plots of standard solutions of urate (range of 2 - 20 mg urate.100ml^{-1}) could be obtained using 1-2m immobilised uricase. The sensitivity of the method was enhanced by using dichlorohydroxyl benzoyl sulphonate as a chromogen in the colour-forming peroxidase reaction. The increased sensitivity enabled the use of the immobilised enzyme tube in the Technicon AA1 AutoAnalyser without any modification to the analytical hardware, for determining the range of urate found in serum.

Operational and storage stability studies showed the feasibility of using the immobilised uricase in routine analysis of urate in clinical laboratories. Linearity of calibration plots could be maintained up to 14 days when the immobilised enzyme tubes were exposed to room temperature, but the life could be prolonged if the enzyme tubes were stored at 4°, a normal requirement for most soluble enzymes and analytical reagents.

The precision of the method presented was high and comparable to many chemical and enzymatic methods (23,38). Depending on the degree of precision required, a sampling rate of 40 or 60h^{-1} could be adopted.

Recovery studies showed that using protein-free standard

solutions, the recovery of added urate to known urate samples was virtually 100%. On the other hand, the recovery of urate was less than 100% when the calibration was carried out with standard solutions of urate containing 7% albumin. Protein binding of urate has been pointed as the cause of reduced dialysis of urate across dialysis membrane, resulting in slight depression in urate values (about 5%)(34). Haeckel (36) also showed that protein was required in standard solutions for higher recoveries of added urate, but samples were not subject to dialysis.

There seemed to be no dialysis effect in these studies, but there was an apparent increase in the gradient of the calibration plot when protein-containing standard solutions were assayed. It was observed in the interference studies, that albumin actually caused a slight elevation in the urate levels. Protein may cause greater sample to sample interactions which may cause increased readings.

Ascorbate has inhibitory effect on uricase (57), and enzymatic determination of urate can be affected by high concentration of ascorbate. However, the interference effect may have been on the colour-forming reactions. Cysteine and glutathione have not been shown to be inhibitory to uricase, so their effects would be on the peroxidase catalysed reactions. Fortunately, possibly due to a binding effect, the interference of cysteine and glutathione could be eliminated. In the case of ascorbate the interference was reduced in the presence of protein and as the normal level of ascorbate in serum is low (less than 2 $mg.100ml^{-1}$), the effect

would be minimal.

Sample interaction was low and as expected lower with the lower sampling rate. Variations in the carry-over coefficients may be due to the cleanliness of the flow system (pumping lines, glass connections and dialysis membrane). In any case the carry-over coefficients were less than 5%.

The presence of NaCl in the buffer was necessary for efficient dialysis or urate and minimisation of sample interactions. Increasing salt concentration, however, did not seem to affect (decrease) the sample interaction, a factor which may be attributed to the absence of charges on the support (of the immobilised enzyme derivative). Slight suppression of the sensitivity with high salt concentration may be due to the "salting out" of oxygen in the buffer solution (working buffer).

Detergent is generally essential in minimising sample interactions. However, it was found that there was no obvious effect in the concentration range of Triton X100 studied. Nevertheless, there was the possibility of the washing effect of the detergent in long continuous operation. In the complete absence of Triton X100 in the assay buffer, the dialysis of urate through the membrane may be affected, though this property was not consistently observed. The state of the dialysis membrane may be the cause for this variation.

The accuracy of this method was compared with that of a soluble uricase method. Statistical data indicated a positive

correlation between the results from the two methods. The data showed a slight bias between the two methods with the soluble method showing about 0.3% lower values.

Lower values of urate were obtained when Wellcomtrol serums were assayed by the immobilised enzyme method. This was to be expected as the manufacturer's data was obtained by the chemical chelation method (21).

Filippusson et al. (106) developed an automated method for the determination of urate using small packed bed of uricase immobilised to nylon powders. The method could be used for estimating urate in the range of 0.01 - 0.1mM (0.17 - 1.7 mg.100ml^{-1}), but no stability and performance data were given.

Dritschilo and Weibel (140) immobilised uricase to porous glass beads producing fairly stable enzyme derivatives, for use in the analysis of urate in biological fluids. The immobilised enzyme was used as a packed bed reactor incorporated into a specially designed analytical system. Linear calibration plots were obtained for concentration of urate up to 1.0mM (17 mg.100ml^{-1}).

Uricase immobilised to the surfaces of microporous membrane was also used for analysis of urate (141). The method utilised centrifugation to force samples through the membrane and thus into contact with the enzyme. It was claimed to have good reproducibility and accuracy, but no stability data were presented.

7. GENERAL DISCUSSION

Bioaffinity chromatography has been shown to be a very useful procedure for uricase purification, a single bioaffinity step resulting in approximately 80-fold purification of the enzyme.

While bioaffinity chromatography can, in theory, be applied to crude enzyme extracts, there are technical problems resulting in difficulties in separating the enzyme-bound affinity supports.

There was some degree of non-specific adsorption on the affinity support, which on accumulation could have masked the ligands and reduced the effectiveness for bioaffinity adsorption. The low specific activity enzyme extract may further promote non-specific adsorption. Non-specific binding (hydrophobic and electrostatic) may occur in bioaffinity process, either with the ligands, spacers or the supports (142). Hjerten et al. (143) in fact utilised hydrophobic affinity chromatography techniques using alkyl and aryl derivatives of agarose, for separation of serum proteins, enzyme extracts and virus particles.

The use of hydrophilic spacer between the support and ligand may diminish or eliminate completely non-specific adsorption (144). Considering that there are many other strong uricase inhibitors (145,146) and support matrices, there are possibilities for producing different bioaffinity supports for uricase separation

utilising the varied chemistry available for coupling ligands and supports (67-71).

General ligand bioaffinity chromatography support as developed by Mosbach et al. (147) should also be considered for enzymes of the purine biosynthetic and catabolic pathways. Many of the enzymes catalyse substrates or are inhibited by compounds comprising the purine ring, and thus there is a possibility of producing a general bioaffinity support for these enzymes, utilising this property.

In summary, the use of high specific activity enzyme extracts from bacterial or fungal sources may necessitate fewer purification steps.

Purified uricase has been successfully immobilised onto nylon with sufficient activity and stability for its use in the enzymatic analysis of urate, in place of its soluble counterpart. The use of bioaffinity chromatography may be useful in the production of large quantity of pure uricase, but it is obvious that the use of enzyme in the immobilised form should make considerable economies in the amount of enzyme used per analysis. It is ironic that, in the development of the analytical system, substrate retardation by the immobilised enzyme drivative was not desirable during analysis (to eliminate sample interaction), whereas this retarding effect [viz. enzyme retardation by immobilised substrate (or analogue)] was essential for protein separation (in bioaffinity technique).

The analytical method presented necessitated the use of soluble peroxidase (POD) in the colour forming reactions. Primary investigations on the immobilisation of POD onto nylon tube showed that the enzyme derivative was operationally unstable (148). A fully enzymatic method linking immobilised uricase to immobilised catalase and alcohol or aldehyde dehydrogenase may be possible as these enzymes have been immobilised to nylon tube (92,149). Direct detection of oxygen consumption may also be adopted employing a flow through oxygen electrode system (90).

There is every possibility that uricase immobilised to nylon tube can be incorporated into other existing analytical hardwares with or without prior modification to these hardwares. Glucose oxidase immobilised to nylon tube has been incorporated into the Technicon All AutoAnalyser successfully (150). Leon et al. (151) used tabular enzyme reactors on the Technicon 'SMAC' High Speed Analyser for the analysis of enzyme substrates with promising results.

Uricase is located strictly in the peroxisomes (52) and this distributive specificity is advantageous in the use of this enzyme as a marker for studying the mechanism of biogenesis, turnover and physiological role of these organelles. The availability of highly purified uricase is essential for the molecular enzymology studies of uricase. Immobilised uricase may provide more information towards the catalytic and molecular properties of uricase. Even the support-ligand complex can be

used as a tool for mechanistic studies of a particular enzyme (152).

The availability of highly purified uricase and its immobilised derivatives may prove useful in the therapeutic treatment of hyperuricaemic diseases, and the low cost factor in using immobilised uricase in analytical operation may enable monitoring of these diseases to be carried out more widely and economically.

The studies of immobilised enzymes may ultimately enable man to harness these biological catalysts for use in rectifying the errors of nature and in man-made industries, incorporating all the conditions necessary for high efficiency functioning.

List of Suppliers

1.	Amicon Ltd.,	57 Queens St., High Wycombe, Bucks.
2.	Aldrich Chemical Co.,	The Old Brickyard New Road, Gillingham, Dorset, SP8 4JL.
3.	B.D.H. Chemical Co.,	Poole, Dorset.
4.	Beckman Instrument Ltd.,	Glenrothes, Fife.
5.	Boehringer Corporation (London) Ltd.,	Bell Lane, Lewes, East Sussex, BN7 1LG.
6.	Cambrian Chemical Ltd.,	Beddington Farm Road, Croydon, CRO 4XB.
7.	Devices Instruments Ltd.,	101 McTaggart Road, Cumbernauld, Dunbartonshire.
8.	Fison Chemical Co. Ltd.,	Bishop Meadow Road, Loughbrough, Leicestershire.
9.	Kipp & Zonen (NL), UK Agent, FT Scientific Instruments Ltd.	Station Industrial Estate, Bredon, Nr. Tewkesbury, Gloucestershire, GL20 7HH.
10.	MSE Scientific Instrument,	Manor Royal, Crawley, West Sussex, RX10 2QQ.
11.	Pharmacia (G.B.) Ltd.,	Paramount House, 75 Uxbridge Road, London W5 5SS.
12.	Portex Ltd.,	Hythe, Kent, CT21 6JL.
13.	PYE Unicam Ltd., Philips Analytical Dept.,	York St., Cambridge, CB1 2PX.
14.	Radiometer A/S 72,	Copenhagen NV, Denmark.
15.	Shandon Scientific Co. Ltd.,	65 Pound Lane, Willesden, London, NW10.
16.	Sigma London Co. Ltd.,	Fancy Road, Poole, Dorset, BH17 7WH.

17.	Technicon Corporation	Tarrytown, N.Y.
18.	The Scientific Instrument Centre Ltd.,	1 Leeke St., London W.C.1.
19.	Wellcome Reagent Ltd., Wellcome Research Lab.,	Beckenham, Kent, BR3 3BS.
20.	Whatman Lab Sales Ltd.,	Springfield Mill, Maidstone, Kent, ME14 2LE.
21.	Yellow Spring Instrument Co.,	Yellow Spring, Ohio, 45387.

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